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Gunawardena, Harsha

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A CLINICAL AND SEROLOGICAL STUDY OF ADULT AND JUVENILE IDIOPATHIC INFLAMMATORY MYOPATHY

Dr. Harsha Gunawardena MBChB MRCP

A thesis submitted for the degree of Doctor of Philosophy

University of Bath
School for Health

September 2010

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For my mother
GLOSSARY

List of abbreviations

A. Methods

AMP Ammonium persulphate
BS3 Bis(sulphosuccinimidyl)suberate
dFCS dialysed Foetal Calf Serum
DMP Dimethyl pimelimidate-2 HCL
DMSO Dimethyl sulfoxide
EDTA Ethylenediaminetetraacetic acid
ELISA Enzyme-linked immunoabsorbant assay
FCS Foetal Calf Serum
FITC Fluorescein isothiocyanate
HCL Hydrogen chloride
HEp-2 Human epithelial cell line
IB Immunoblotting
IIF Indirect immunofluorescence
IPP Immunoprecipitation
K562 Human chronic myelogenous leukaemic cell line
MALDI-TOF Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry
MBq Megabecquerel
NaCl Sodium Chloride
NaHCO₃ Sodium Bicarbonate
PBS Phosphate-buffered Saline
RPMI Rosewell Park Memorial Institute 1640 medium
SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS Tris-buffered Saline
TEMED N,N,N¹,N¹-tetramethylethylenediamine
Tris Tris[hydroxymethyl]aminomethane
WB Western Blotting
### GLOSSARY

#### List of abbreviations

**B. Clinical**

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<tr>
<td>AIP</td>
<td>Acute interstitial pneumonia</td>
</tr>
<tr>
<td>ANA</td>
<td>Anti-nuclear autoantibody</td>
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<tr>
<td>ANoA</td>
<td>Anti-nucleolar autoantibody</td>
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<tr>
<td>AOMIC</td>
<td>Adult Onset Immunogenetic Collaboration</td>
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<tr>
<td>ARS</td>
<td>Aminoacyl-tRNA</td>
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<tr>
<td>ASS</td>
<td>Anti-synthetase syndrome synthetases</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>ATS</td>
<td>American Thoracic Society</td>
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<tr>
<td>BIRD</td>
<td>Bath Institute for Rheumatic Diseases</td>
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<tr>
<td>CADM</td>
<td>Clinically-amyopathic dermatomyositis</td>
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<tr>
<td>CAT</td>
<td>Cutaneous Assessment Tool</td>
</tr>
<tr>
<td>CHAQ</td>
<td>Childhood Health Assessment Questionnaire</td>
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<tr>
<td>CK</td>
<td>Creatinine kinase</td>
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<td>CMAS</td>
<td>Childhood Myositis Assessment Scale</td>
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<td>COP</td>
<td>Organising pneumonia</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CTD</td>
<td>Connective Tissue Disease</td>
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<td>DM</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DPLD</td>
<td>Diffuse parenchymal lung disease</td>
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<tr>
<td>EMG</td>
<td>Electromyogram</td>
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<tr>
<td>ENA</td>
<td>Extractable nuclear antigen</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>ERS</td>
<td>European Respiratory Society</td>
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<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
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<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
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<tr>
<td>HLA</td>
<td>Human leucocyte antigen</td>
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<td>HRCT</td>
<td>High-resolution computerised tomography</td>
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<td>IBM</td>
<td>Inclusion body myositis</td>
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<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IIM</td>
<td>Idiopathic inflammatory myopathy</td>
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GLOSSARY

List of abbreviations

B. Clinical

JDM        Juvenile dermatomyositis
JDRR       Juvenile dermatomyositis Registry and Repository
LDH        Lactate dehydrogenase
MAA        Myositis-associated autoantibody
MCTD       Mixed connective tissue disease
MDA5       Melanoma-differentiation associated gene 5
MH         Mechanic's hands
MHC        Major histocompatibility complex
mRNA       messenger ribonucleoprotein
MSA        Myositis-specific autoantibody
NSIP       Non-specific interstitial pneumonia
NURD       Nucleosome remodelling deacetylase
NXP-2      Nuclear matrix protein-2
PBC        Primary biliary cirrhosis
pDCs       Plasmacytoid dendritic cells
PGA        Physician’s global assessment
PM         Polymyositis
RANGAP     Ran GTPase activating protein
RNHRD      Royal National Hospital for Rheumatic Diseases
RP         Raynaud’s phenomenon
SAE        Small ubiquitin like modifier enzyme
SLE        Systemic lupus erythematosus
SNP        Single nucleotide polymorphism
SRP        Signal recognition particle
SSc        Systemic sclerosis
SUMO       Small ubiquitin-like modifiers
TIF1-γ     Transcriptional intermediary factor 1-gamma
TNF        Tumour necrosis factor
tRNA       transfer ribonucleoprotein
UV         Ultra-violet
VAS        Visual analogue score
VCAM-1     Vascular cellular adhesion molecule-1
UIP        Usual interstitial pneumonia
ABSTRACT

**Introduction:** The idiopathic inflammatory myopathies (IIM): dermatomyositis (DM) and polymyositis (PM) have been historically defined by broad clinical and pathological criteria. These conditions affect both adults and children with clinical features including muscle weakness, skin disease and internal organ involvement. Using a clinico-serological approach DM and PM can be defined into more homogeneous subsets. Myositis-specific autoantibodies (MSAs) are directed against cytoplasmic or nuclear components involved in key regulatory intra-cellular processes including protein synthesis, translocation and gene transcription. Over the last few years MSAs have been better characterised including autoantibodies directed against the aminoacyl tRNA-synthetase (ARS) enzymes, the signal recognition particle and the Mi-2 protein.

**Aim:** The overall aim of this thesis is to describe a comprehensive clinical and serological study of adult and juvenile IIM. Autoantigen targets including novel specificities were identified using protein immunoprecipitation.

**Results:** The first part of this thesis is a descriptive study on known myositis autoantibodies in adult IIM, confirming the significant association of interstitial pneumonia with anti-ARS, severe myopathy with anti-SRP, and classic DM with anti-Mi-2 serotype. In the next section, new autoantigen systems in adult IIM are described including a new anti-ARS (anti-Zo) in the anti-synthetase syndrome. Further autoantibodies directed against small ubiquitin-like modifier enzyme and a p155/140 autoantigen are major serological subsets in adult DM, the latter significantly associated with malignancy. The final section outlines a large serological study of juvenile DM (JDM) and JDM-overlap showing the frequency and clinical associations of MSAs and myositis-associated autoantibodies, including work on two new major subsets anti-p155/140 and anti-p140, which appear to define more severe disease.

**Conclusion:** The work in this thesis highlights the importance of autoimmunity in IIM and suggests a new approach where MSAs can classify patients into clinical syndromes, which predict outcomes and may as a result influence treatment strategies.
CHAPTER 1
INTRODUCTION

1.1 Background
The idiopathic inflammatory myopathies (IIM) are a group of rare autoimmune diseases that affect both adults and children. The major subtypes of IIM are polymyositis (PM) and dermatomyositis (DM) that are heterogeneous conditions characterised by skeletal muscle weakness, biochemical or histological evidence of muscle inflammation, skin lesions and systemic organ involvement (1, 2). Morbidity outcomes relate to well-known complications, e.g. cancer-associated myositis and interstitial pneumonia. In children there is also a significant morbidity associated with vasculopathic organ involvement, soft tissue calcinosis and skin ulceration. Response to immunosuppressive therapies is variable and patients may die from disease or treatment complications, or remain very disabled. The associations with cancer and interstitial pneumonia make IIM an important autoimmune model to investigate further. The precise aetiology is unknown, mechanisms require further elucidation but the interaction between immunogenetic and environmental factors appears to play a major role (3). Like other systemic autoimmune diseases including systemic lupus erythematosus (SLE) and systemic sclerosis (SSc), IIM is characterised by the production of autoantibodies. The identification and characterisation of autoantibodies is an important cornerstone in the diagnosis of autoimmune connective tissue disease (CTD) (4, 5). Autoantibodies and corresponding target autoantigens are the subject of considerable interest, and there is increasing evidence to support a critical association between genotype, serotype and clinical phenotype in both adult and juvenile myositis (6-10).

1.2 Classification and diagnosis
In 1975, Bohan and Peter first proposed criteria to aid the diagnosis and classification of PM and DM (1, 2). The five adapted criteria proposed were as follows:

1. Symmetrical proximal weakness of the limb or pelvic girdle and anterior neck flexors progressing over weeks to months, with or without dysphagia or respiratory muscle involvement.
2. Elevation of muscle enzymes, including creatinine kinase (CK).
3. Abnormal electromyogram (EMG) with a triad of
   • Short duration, small amplitude polyphasic motor unit potentials.
- Fibrillations, positive sharp waves, increased insertional irritability.
- Spontaneous, bizarre, high frequency repetitive discharges.

4. Abnormal muscle biopsy demonstrating inflammatory cell infiltrate, muscle degeneration, regeneration or necrosis.

5. Typical cutaneous signs of DM including
- Heliotrope rash – violaceous erythema on upper eyelids
- Gottron’s papules – violaceous keratotic papules on extensor aspects of fingers or elbows or knees.

Exclusion criteria: absence of other forms of myopathy e.g. inclusion body myositis, metabolic, inherited or infectious forms of myopathy.

Bohan and Peter suggested five subsets of myositis: PM, DM, cancer-associated myositis, juvenile myositis and myositis overlapping with CTD. Bohan and Peter also described that patients can develop cutaneous features of DM, which may precede the onset of myopathy (2).

- Definite DM requires all five criteria.
- Definite PM requires first four criteria (without rash).

- Probable disease requires three criteria out of first 4 criteria (for DM, one of which is rash i.e. criteria 5).

- Possible disease requires two criteria out of first 4 criteria (for DM, criteria 5 plus one other).

Overlap myositis: must fulfil criteria for their main CTD e.g. systemic sclerosis (11) or systemic lupus erythematosus (12), at least two of criteria 1 – 4, and at least one myositis-specific or associated autoantibody (see section 1.6.4).

Tanimoto et al published further classification criteria for PM / DM in 1995 (13).

1. Skin lesions:
   - Heliotrope rash.
   - Gottron’s papules (extensor aspect of fingers, elbows or knees.

2. Systemic inflammatory signs (elevated erythrocyte sedimentation rate, C-reactive protein, pyrexias, weight loss).

3. Non-erosive arthritis.
4. Myositis-specific autoantibodies e.g. anti-JO-1
5. Elevated skeletal muscle enzymes.
6. Myalgia at rest or with contraction.
7. Proximal muscle weakness.
8. Myopathic changes on EMG.
9. Myositis changes on muscle biopsy.

- For a diagnosis of definite PM – at least four from item list 2 – 9.
- For a diagnosis of definite DM – at least four from item list 2 – 9 plus at least one feature from item 1.

The IIM spectrum also includes a further subset, sporadic inclusion body myositis (IBM) and Griggs et al have subsequently proposed classification criteria (14). Unlike other IIM, sporadic IBM causes slowly progressive myopathy with atrophy with a distinctive pattern of muscle involvement that may be asymmetrical and affect distal rather than proximal muscle groups, and is often unresponsive to conventional immunomodulatory therapy (15). Historically, IBM cases have been seen and managed by Neurologists. Clinicians now recognise histopathology and other more specific investigations are required to exclude IBM, hereditary and other non-immune mediated myopathies when applying the Bohan and Peter criteria. This study has not investigated this subgroup of patients and in this thesis IIM refers purely to PM and DM cases.

1.3 Epidemiology
The true frequency of PM and DM as stand-alone disorders or in association with other CTD is unknown. Reports of incidence and prevalence are limited based on different study populations. Estimates based on the diagnostic criteria proposed by Bohan and Peter (1, 2), may not be accurate due to difficulties distinguishing PM and IBM. Reports show an annual incidence of 2-7 / million population (16-18). The mean age of onset is 50 years affecting women more than men. Juvenile dermatomyositis (JDM) is the most common IIM of children and the reported incidence is 0.8-4.1 per million children per year (18-20). In contrast to adults, juvenile PM is rare (21), whereas JDM overlap with other connective tissue disease in particular SSc and SLE is more common.
1.4 Clinical features
IIM is characterised by a spectrum of clinical features including skeletal muscle weakness, skin lesions and systemic organ involvement, in particular interstitial pneumonia and a risk of cancer in adults. Children with JDM share some clinical features with adult patients in terms of muscle disease and characteristic skin lesions. Certain clinical features including skin ulceration, calcinosis and gastrointestinal involvement are more common in children and have been proposed as predictors of severe disease course in JDM (21-25). In contrast to adults with DM, both interstitial pneumonia and cancer-associated myositis are very rare in JDM. Delays in diagnosis, in particular recognising active muscle or lung disease and poor response to treatment mean that patients are often left disabled from irreversible damage.

1.4.1 Myositis
IIM can present with progressive symmetrical muscle weakness that usually develops over a few weeks to months. The proximal muscle groups such as the shoulder girdle, pelvic girdle, thigh muscles and neck flexors are typically involved. In severe cases, other muscle groups can be involved including chest wall weakness, which can compromise respiratory function. At onset, patients rarely complain of muscle pain but experience difficulties with activities of daily living, for example, rising from a chair, walking up the stairs and raising their arms. Depending on the severity of the case and the response to treatment muscle atrophy can develop that leads to severe irreversible weakness.

1.4.2 Cutaneous manifestations
Classic DM skin lesions are generally present at the time of diagnosis and in some cases may precede the development of muscle inflammation or other organ involvement (see Section 1.5.3) (26). Gottron’s sign is an erythematous, scaly plaque-like lesion usually found over the dorsal surface of the metacarpophalangeal or proximal interphalangeal joints and the extensor surfaces of the knees or elbows. Heliotrope rash is a purple violaceous eruption of the upper eyelids, often with cutaneous oedema. More extensive skin involvement includes the V-sign and Shawl-sign rash over the anterior triangle of the neck, and the back of the neck and shoulders respectively. Erythroderma can develop on the face (malar distribution or forehead). All these rashes are diffuse, flat and erythematous and may be photosensitive. The skin lesions can have a poikilodermatous appearance with a combination of atrophy, pigment changes and telangiectasia. Periungual erythema
with abnormal dilated nailfold capillaries is another feature. Mechanic's hands are a characteristic cutaneous manifestation that is pathognomonic of the clinical and serological subset termed the anti-synthetase syndrome (see Section 1.5.1). The description refers to dry, cracked or fissured skin with hyperkeratosis over the lateral aspects of the inter-digits especially the index and middle fingers. Certain features secondary to persistent cutaneous vasculitis are more common in JDM, in particular generalised oedema, skin ulceration and soft tissue calcification (calcinosis) (21-25).

1.4.3 Interstitial pneumonia

Diffuse parenchymal lung disease (DPLD) is a major cause of morbidity and mortality in IIM (27). Primary lung involvement or ventilatory failure secondary to muscle weakness is common, more so in adult IIM (28-30). Interstitial pneumonia is the most frequent sub-type of DPLD in IIM, with studies showing an overall frequency of between 20-60% (29, 31). Interstitial pneumonia (also termed interstitial lung disease) are a heterogeneous group of non-malignant and non-infectious lung disorders that comprise of a number of clinico-pathological entities that are sufficiently different to be designated as separate diseases. The characteristic hallmark is that of varying patterns of inflammation and fibrosis affecting the lung parenchyma.

In 2002 the American Thoracic Society and the European Respiratory Society (ATS/ERS) published the consensus classification for interstitial pneumonia that is equally applicable in CTD (32). Based on radiological and histopathological patterns, interstitial pneumonia can be further subdivided into several subsets including usual interstitial pneumonia (UIP), non-specific interstitial pneumonia (NSIP), organising pneumonia (OP) and acute interstitial pneumonia (AIP), which are recognised in CTD (32, 33). The histological hallmark of UIP is the presence of fibroblastic foci with a subpleural, basal and peripheral distribution (32, 34). The most important differential pattern of UIP, particularly in CTD is NSIP, which has a similar subpleural and basal distribution. Katzenstein et al first described NSIP characterised by cellular and fibrotic changes with the absence of dense fibrosis or fibroblastic foci (35). With the advent of high-resolution computerised tomography (HRCT), interstitial pneumonia patterns in CTD can be categorised radiologically with good histological correlation (36, 37).

NSIP has been reported to be the most frequent subtype on both HRCT and lung biopsy in IIM (38). AIP, COP and UIP have also been reported and identified as
patterns responsible for acute or rapidly progressive interstitial pneumonia with AIP and UIP associated with the worst prognosis (28-31, 33, 39). It is well recognised that interstitial lung involvement may be the first or predominant organ manifestation preceding muscle or skin disease, particularly in the anti-synthetase syndrome (see Section 1.5.1) and in patients with clinically-amyopathic DM (CADM) (see Section 1.5.3) (29, 31, 40-42).

1.4.4 Arthritis and overlap features
Non-erosive inflammatory arthritis is a frequent manifestation of IIM, particularly in anti-synthetase syndrome and overlap conditions such as SSc, mixed connective tissue disease and SLE. Raynaud’s phenomenon characterised by vasospasm and structural changes of the digital microvasculature is a common feature. Joint disease with contractures and lipoatrophy of soft tissues is more frequent in JDM particularly in patients with overlap scleroderma. General features include fatigue, weight loss and fever.

1.4.5 Other organ manifestations
Other organs can be involved including the striated muscles of oropharyngeal and upper oesophagus causing dysphagia, and gastro-intestinal tract vasculopathy leading to ischaemic ulceration with pain and bleeding, the latter a more common complication in JDM (43). Cardiac involvement including myocarditis has been reported with subsequent conduction defects and ventricular failure (44, 45). Although rare, cardiac disease confers a high risk for mortality in patients with IIM (27, 44).

1.5 Specific clinical subsets
1.5.1 Anti-synthetase syndrome
The anti-­a­minoacyl tRNA synthetase (anti-ARS) autoantibodies (see Section 1.6.2) define the anti-synthetase syndrome recognised as a spectrum of myositis, interstitial pneumonia, non-erosive arthritis, fever, Raynaud’s phenomenon and mechanic’s hands (46). Patients may have different clinical features within the syndrome, but overall there is a significant association with interstitial pneumonia and studies have described the frequency to be as high as 95% (28-30, 47, 48).
1.5.2 Cancer-associated myositis

The association between cancer and adult DM is well recognised particularly in the older age groups (49-54). Because IIM can have a chance association with malignancy, cancer-associated myositis is defined by a temporal association of less than three years between cancer and DM onset (50, 51). The association with PM is uncertain with some studies reporting an increased relative risk but some not (53, 55, 56). Hill et al conducted a pooled analysis of published national data from Sweden, Denmark, and Finland on all patients with DM and PM (over the age of 15 years). The study calculated standardised incidence ratios (SIR) for individual cancer sites for dermatomyositis and polymyositis separately, using national cancer rates by country, sex, age, and date. The frequency of cancer in the DM cohort was 32% (58% of which developed cancer after the diagnosis of DM), with a SIR of 3.0. The most common cancer observed was ovarian followed by lung, pancreatic, non-Hodgkin lymphoma stomach and colorectal. 15% of PM cases were associated with cancer (70% developed after the diagnosis of PM), with a SIR of 1.3. PM was associated with a higher risk of non-Hodgkin lymphoma, then lung and bladder malignancy. In both DM and PM, the risk of malignancy was highest at the time of myositis diagnosis (within the first year) with a markedly reduced relative risk over time. For DM the SIR of cancer by year after diagnosis of myositis was 13.5 (0-1 year), 2.5 (2-5 years) and 1.4 (>5 years). In the PM group the SIR of cancer by year after diagnosis of myositis was 2.6 (0-1 year), 1.5 (2-5 years) and 0.9 (>5 years). In the DM cohort the number of cancers recorded in patients under the age of 44 years was small, and there was no increase in risk in PM patients (53). In contrast to adults, JDM appears not to be associated with malignancy (53, 57). Historically, cancer-associated myositis has not been associated with specific myositis autoantibodies, but recent reports including work described in this thesis have described a novel autoantibody marker in this clinical subset (see section 1.6.2).

1.5.3 Clinically-amyopathic dermatomyositis (CADM)

Patients may only develop DM skin changes with no clinical evidence of myopathy, termed DM sine myositis or amyopathic DM (ADM) (40). Clinically-amyopathic DM (CADM) is a designation for patients who either have ADM or those patients who have DM skin lesions, no muscle weakness but evidence of sub-clinical myopathy on further testing (muscle enzymes, EMG, imaging or biopsy), termed hypomyopathic DM.
1.6 Treatment
The treatment of both PM and DM is often empirical and in general is influenced by clinical experience rather than robust clinical data. There remains a paucity of standardised clinical trials due to the relative rarity of PM and DM. In addition, perhaps due to historical classification tools including the Bohan and Peter criteria, IIM is a heterogeneous condition, which makes designing trials and interpreting treatment outcomes in different disease subtypes difficult. For example, optimal treatment strategies for ASS with lung disease may be different to other subgroups, CADM or immune-mediated necrotising myopathy. Moreover, the absence of standardised disease activity and outcome measures has made the assessment of treatment response difficult. Classifying patients into more homogenous subsets based on serotype / clinical phenotype, and the advent of validated myositis disease activity tools (see section 1.7) will facilitate the design of clinical trials, which can capture clinically significant changes.

Based on personal clinical experience and limited trial data there are several treatments that are effective treating patients with IIM.

Glucocorticoids
Prednisolone is recognised as the first line treatment for PM or DM including those with internal organ complications e.g. ASS with interstitial lung involvement. This is primarily based on clinical experience rather than trial data. In the majority of cases, the initial dose is 0.5-1mg/kg daily, and depending on clinical response the dose is weaned over a number of months. Patients may remain on low dose prednisolone for a number of years, particularly those with ASS and ILD, which appears to be very steroid responsive but also steroid dependant with disease flares on steroid withdrawal (48) (personal observation). In severe cases of myositis with internal organ involvement (including those with gastrointestinal involvement e.g. vasculopathy or pharyngeal dysfunction), patients may require induction with intravenous methylprednisolone pulses.

Specific immunomodulatory therapy
In patients who respond to steroids, the goal is to induce disease control and thus remission as quickly as possible at the same as minimising steroid requirements. In mild to moderate cases, for example those with purely muscle and / or skin disease, this may be achieved with either Azathioprine or Methotrexate (58-61). An alternative to Azathioprine and Methotrexate is Ciclosporin, the latter two agents
have also been compared in a small randomised controlled trial, with no significant difference observed, although appeared effective (62). However, the side effect of profile may be higher with Ciclosporin, which often limits its use in clinical practice. In moderate to severe cases, induction therapy in combination with pulsed / oral prednisolone may be required. The efficacy of intravenous immunoglobulin (IV IG) has been demonstrated, especially in DM, in a randomised controlled trial (63). In DM patients who response to standard regimes with prednisolone and Azathioprine / Methotrexate is inadequate or in those patients with severe disease at presentation (not complicated by interstitial pneumonia), IV IG is the preferred agent of choice. Repeated infusions may be required to achieve disease control followed by maintenance therapy as outlined above. In severe cases, including those with significant interstitial pneumonia and / or severe necrotising myopathy, based on personal clinical experience, IV Cyclophosphamide (CYC) may be a useful adjuvant therapy. In general, induction with 4-8 pulsed infusions given every 3-4 weeks is an optimal strategy. If this treatment fails or is contra-indicated, the novel biologic agent Rituximab (monoclonal antibody against CD20-positive B cells) appears to be an attractive treatment option, especially in myositis-autoantibody-positive refractory patients e.g. ASS patients with interstitial pneumonia and DM (64). A multi-centre / international randomised placebo controlled has recently completed recruitment (http://www.edc.gspn.pitt.edu/rimstudy/index.html). There is now preliminary data for newer agents in moderate disease or as maintenance therapy following induction with IV IG, IV CYC or Rituximab. Mycophenolate Mofetil inhibits the de novo pathway of nucleotide synthesis leading to an anti-proliferative affect directed against T and B lymphocytes. It appears to be a useful agent particularly in those patients where Azathioprine or Methotrexate is ineffective or poorly tolerated. There is also preliminary data that suggests Mycophenolate may be beneficial in refractory IIM cases associated with interstitial pneumonia (65-67). A further option is Tacrolimus, a calcineurin inhibitor that blocks T cell signal transduction and IL-2 signalling. This drug can be effective in some difficult-to-treat cases of polymyositis / ASS, especially in patients with interstitial lung disease (68, 69).

1.7 Assessment Tools

In a clinical setting, proximal myopathy is often assessed by physician tested medical research council (MRC) scale for muscle strength. Manual muscle testing, however, has a number of limitations. One limitation is that the MRC scale is an ordinal scale with disproportional distances between grades. Another limitation of the MRC scale is that the scoring depends on the judgment of the examiner. Finally,
with the 6-point ordinal MRC scale, it is difficult to identify relatively small but clinically relevant changes in muscle strength. Alternative more objective tests to assess proximal muscle function have been devised, including two isotonic tests (1 kg arm lift for upper limb function and a 30 second chair stand test for lower limb function. These tests exhibit excellent test-retest reliability, demonstrate construct validity and are responsive to changes in disease activity in IIM (70). New tools have been devised to standardise the conduct and reporting on IIM clinical trials. Two tools, known as the myositis intention to treat index (MITAX) and the myositis disease activity assessment visual analogue scale (MYOACT), have been developed to measure activity in patients with IIM (www.niehs.nih.gov/research/resources/collab/imacs/docs/activity/MDAAT_Scoring_2009.pdf). In addition, the myositis damage index (MDI) has been devised to assess the extent and severity of damage developing in different organs and systems. These measures have been reviewed by the International Myositis Assessment and Clinical Studies (IMACS) group and have been found to have good face validity (71). A recent study has demonstrated that the myositis disease activity assessment tool (MITAX and MYOACT) has good interrater reliability and validity in clinical practice (72). In addition to these tools, specific disease assessment scales have also been designed in juvenile IIM, which are used in routine clinical practice, as well as research studies (see section 5.1.2 and appendix 5.5).

1.8 Pathogenesis and aetiology
It is becoming increasingly clear that IIM is a useful model to study the relationship between immunogenetic profiles, autoimmune targets and clinical phenotype in CTD. The development of IIM appears to be secondary to the combination of genetic factors and exposure to environmental agents that may initiate specific autoimmune responses in certain individuals.

1.8.1 Pathogenesis

Classic descriptions
Cellular and humoral autoimmune mechanisms that target skeletal muscle, the lungs, the skin, and associated microvasculature are implicated in the pathogenesis of IIM. In broad terms, in DM, immune responses are said to primarily target the microvascular endothelium leading to activation of the complement cascade, upregulation of cytokines and chemokines, and thus endothelial damage. Inflammatory lesions with B and CD4 positive T cells are predominately found in perivascular and perimysial regions with perifascicular atrophy. Endothelial
hyperplasia and fibrin thrombi are seen in both endomysial and dermal capillaries (17, 73, 74). Cytokines are released, which leads to upregulation of vascular-cell adhesion protein-1 (VCAM-1) and intercellular adhesion molecule (ICAM-1), which promotes endothelial damage. Historically in PM, myocytotoxicity is said to be mediated by cellular responses where clonally expanded CD8 positive T cells are primed against muscle fibres expressing MHC class I antigens leading to an inflammatory cell infiltrate that invades muscle fascicles (17, 75). Previous descriptions have suggested B cells are sparse within the inflammatory cell infiltrate of PM specimens (76).

**Shared cellular and humoral mechanisms**

It is now becoming increasingly clear that both T and B cell responses occur in both subtypes of IIM. Both CD4 and CD8 T cells have been demonstrated in PM and DM muscle tissue (77). In addition, cytokines, chemokines and their receptors, are strongly expressed by endothelial and inflammatory cells in both subtypes. CD4+ cells previously characterised as T cells have been identified as plasmacytoid dendritic cells (pDCs). Large-scale gene expression studies have confirmed a type 1 interferon (IFN) signature with abundant pDCs in DM muscle and skin (78, 79). More recently, type 1 IFN α/β producing pDCs have also identified in PM muscle (80), highlighting the potential role of this cytokine system in the pathogenesis of IIM. Furthermore, antigen-driven B cell humoral responses have now been demonstrated within muscle in PM, as well as DM. A previous study has shown that immunoglobulin transcripts are prominent in PM muscle, with a relative abundance of CD138+ plasma cells (81). Further work has confirmed local B cell maturation occurs within myositis muscle in both subtypes of IIM; B cells and corresponding plasma cells were clonally expanded, had class-switched and undergone mutation (82). This suggests antigen-driven responses may play a crucial role in disease pathogenesis. The role for B-cell autoimmunity in PM and DM is further emphasised by the production of autoantibodies against specific intra-cellular protein targets, including the aminoacyl-tRNA-synthetases, signal recognition particle and Mi-2.

**Non-immune mechanisms**

It has been suggested that non-immune mechanisms may play a role in disease pathogenesis. The rationale being some patients can have minimal inflammatory cell infiltrate but marked structural changes on muscle biopsy with significant weakness. In a mouse model, over-expression of class I MHC molecules has been shown to mediate skeletal muscle fibre damage, even in the absence of T and B
cells (83). Similarly, in human myositis, induction of class I MHC in muscle fibres occurs before mononuclear cell infiltration (84, 85). Recent data suggests continuous upregulation of MHC class I on muscle fibers leads to an endoplasmic reticulum (ER) stress response. A potential mechanism has been proposed whereby ER overload leads to activation of nuclear factor kappa-B pathway combined with the ER unfolded protein response, which in turn promotes pro-inflammatory cytokine activity, cell death and muscle fibre necrosis (86).

1.8.2 Immunogenetics
Evidence for a genetic basis for IIM has largely accumulated through candidate gene studies, although a familial predisposition is suggested from case reports of familial clustering (87). Candidate gene studies have confirmed that HLA-DRB1*0301 and HLA-DQA1*0501 are definite risk factors for the development of Caucasian IIM (9, 87-89). These alleles form part of the conserved, ancestral Caucasian haplotype, A1-B8-Cw7-DRB1*0301-DQA1*0501. Recent studies have confirmed IIM in different ethnic groups are associated with different HLA-DR and DQ alleles (8, 89-91).

Major histocompatibility complex (MHC) differences exist between the traditional clinical PM and DM sub-types, where HLA-DRB1*07 confers risk for DM but is protective for PM (9). The 8.1 ancestral MHC haplotype, (HLA-B*08-DRB1*03-DQA1*05-DQB1*02) is associated with adult and juvenile IIM (9, 10), where the strongest signal appears to come from the HLA class I region (89, 92). Furthermore, TNF-308A single nucleotide polymorphisms (SNP) are associated with IIM, but only due to strong linkage disequilibrium with HLA-B*08, thus forming part of an extended 8.1 haplotype (92). Clinical phenotype is more strongly associated with myositis autoantibodies than with traditional clinical sub-type in adult Caucasian IIM (9, 89). HLA class II haplotype (DRB1-DQA1-DQB1) is in turn associated with antibody status. For example in adults, the DRB1*07-DQA1*02-DQB1*02 haplotype is strongly associated with anti-Mi-2, whereas the 8.1 haplotype is strongly associated with anti-Jo-1 autoantibodies (see also Section 1.6.2) (9).

1.8.3 Environmental factors
Environmental triggers may also influence the development of myositis. Evidence implicating potential environmental factors includes the observation that seasonal patterns are associated with the development of specific IIM-serological syndromes e.g. anti-Jo-1 positive patients have been shown to develop disease in the Spring
In a recent study, seasonal birth patterns in myositis subgroups suggested an aetiological role of early environmental exposures (95). In particular, birth distributions appeared to have a stronger seasonal association in JDM, suggesting a potential role for specific perinatal exposure.

There is evidence to suggest an aetiological role of ultra-violet (UV) light in the pathogenesis of autoimmune CTD. From a clinical perspective SLE and DM are associated with photosensitive rashes, and disease flares can be induced by sunlight exposure. Studies have demonstrated that UV-B light induces apoptosis of keratinocytes, which leads to the redistribution of certain intracellular proteins that are classically recognised by SLE-associated autoantibodies (96-98). In particular, autoantigens were shown to be clustered into two distinct populations, either in surface blebs or larger surface bodies on apoptotic cells (96). Apoptotic blebs contained cytoplasmic components including fragmented endoplasmic reticulum and the Ro52 protein. Apoptotic bodies contained nuclear associated autoantigens, nucleosomal DNA, Ro60, La, and the small nuclear ribonucleoproteins. These findings implicate apoptotic cells as an important potential source of concentrated or modified autoantigens that may drive autoantibody production and skin disease in SLE (97). Similar to SLE, there is evidence to suggest an aetiological role of UV-light in the pathogenesis of DM. Firstly, the incidence of DM cases has been shown to be higher in countries with a latitudinal gradient towards the equator (99). Secondly, surface UV-radiation intensity has been shown to be associated with the relative frequency of DM cases, especially those with anti-Mi-2 autoantibodies (classically associated with hallmark cutaneous DM lesions, see section 1.6.4.1). Collectively, this data provides insight into the possible aetiological mechanisms of the development of DM.

1.8.4 Autoantibodies and targeted autoantigens
The identification and characterisation of autoantibodies is an important cornerstone in the diagnosis of autoimmune CTD (4, 100, 101). Autoantibodies target nuclear or cytoplasmic intra-cellular components and specific profiles are associated with clinical phenotypes within the CTD disease spectrum. Autoimmunity is characterised by the loss of tolerance to self-proteins or autoantigens. In recent years there have been several attractive paradigms proposed to explain why certain seemingly ubiquitously expressed proteins are selectively targeted by autoantibodies. Autoantigens appear to be critical partners in driving autoreactivity, unified by their susceptibility to cleavage by certain apoptotic substrates. Studies have
demonstrated the protease granzyme B, an enzyme released by cytotoxic T cells, generates a unique form of apoptosis that leads to the cleavage of certain intracellular proteins into novel fragments. These fragments become clustered and may trigger autoreactive responses. This mechanism has been strongly implicated in the pathogenesis of several autoimmune diseases including SLE, SSc and IIM (96, 97, 102-106). Furthermore, this apoptotic trigger is likely to occur in specific pro-immune microenvironments and there is now emerging evidence to suggest that specific autoantigens are upregulated in different cell types and lesional tissues, particularly in IIM (107, 108).

1.8.4.1 Autoantibodies in myositis

Previous studies have shown that autoantibodies are detected in approximately 50-60% of IIM patients depending on the detection methods used (46, 109, 110). The standard techniques have included immunofluorescence on human-epithelial cell lines (HEp-2 cells), generic or antigen-specific ELISA tests, immunodiffusion and counter-immunoelectrophoresis. More specific techniques including immunoblotting and in particular protein radioimmunoprecipitation (IPP) have the advantage of being able to detect novel autoantigen targets. The major advantage of IPP is that it identifies those autoantibodies that target conformational epitopes. In addition, novel targets are amenable to identification using a proteomic approach. The identification and characterisation of novel autoantibody subsets in IIM in this study are based on the method of IPP.

Autoantibodies in IIM can be divided broadly into two groups, myositis-specific autoantibodies (MSAs) or myositis-associated autoantibodies (MAAs). MAAs are found in myositis overlap syndromes, in particular SSc, MCTD and SLE. In general, MSAs are unique to PM and DM, and the previously described specificities are outlined below. See Tables 1 and 2, and Figure 1.
**Anti-synthetase autoantibodies**

Autoantibodies against the cytoplasmic aminoacyl-tRNA synthetase enzymes (ARS) are the most frequently detected in adult patients with myositis. The first to be described and most common autoantigenic target is Jo-1 (histidyl tRNA-synthetase) (111), which is found in approximately 20% of IIM patients (112, 113) . Autoantibodies to six other ARS molecules; PL-7 (threonyl), PL-12 (alanyl), EJ (glycyl), OJ (isoleucyl), KS (asparaginyl) and Ha (tyrosyl) have been described collectively in approximately 20% of patients with IIM (frequency of patients with each non-Jo-1 anti-ARS is between 1-5% (114-119). A further anti-ARS termed anti-Zo (phenylalanyl) has been discovered by our group and will be described in Chapter 4 (120). The ARS are a distinct group of enzymes that catalyse the binding of specific amino acids to their cognate tRNA for incorporation into growing polypeptide chains. The anti-ARS autoantibodies define the anti-synthetase syndrome, which has been described in Section 1.5.1.

**Anti-SRP autoantibodies**

Patients with autoantibodies to the signal recognition particle (SRP) appear to form a further distinct clinical and histopathological subset. Reeves et al were the first to report SRP as an autoantigen target in myositis (121). The SRP autoantigen is a ribonucleoprotein complex (proteins-7SLRNA) with the 54 kDa and 72 kDa polypeptides most frequently targeted. The cytoplasmic SRP protein recognises secretory or membrane bound proteins and regulates protein translocation through the endoplasmic reticulum. Anti-SRP autoantibodies are found in approximately 5% of patients with IIM (122, 123). Patients with anti-SRP can present with acute onset severe myopathy with significant muscle enzyme elevation and systemic features including dysphagia that can be refractory to standard treatments (123, 124).

**Anti-Mi-2 autoantibodies**

Anti-Mi-2 is classically detected in adult and juvenile patients with DM clinical features. Anti-Mi-2 is detected in between 5-20% of patients with DM (6, 10, 125-128). Mi-2, a nuclear helicase protein forms part of the nucleosome remodelling deacetylase (NuRD) complex, which plays a role in gene transcription (129). This autoantibody specificity is described in patients with hallmark cutaneous DM lesions including Gottron’s papules, heliotrope rash, cuticular overgrowth and the V-sign / Shawl-sign. Moreover, patients may have milder muscle involvement with a lower risk of interstitial pneumonia and respond well to therapy (6, 125-127).
**Anti-CADM-140 autoantibodies**

A novel autoantibody specificity termed anti-CADM-140 in a Japanese cohort associated with CADM and rapidly progressive interstitial pneumonia (130). This autoantibody specificity was not seen in classic DM, PM or any other CTD cases. Using a series of molecular techniques the CADM-140 autoantigen has recently been identified as the cytoplasmic protein melanoma-differentiation associated gene 5 (MDA5) (131). MDA5 is involved in innate immune responses against viral infections (132).
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<tr>
<th>Autoantibodies</th>
<th>Autoantigen and function</th>
<th>Clinical Phenotype</th>
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<td>Anti-U1-RNP</td>
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## Table 2: Myositis-specific autoantibodies, autoantigens and clinical features

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<th>Clinical Phenotype</th>
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</thead>
<tbody>
<tr>
<td>Anti-ARS</td>
<td>ARS – intracytoplasmic protein synthesis (binding of specific amino acid to its cognate tRNA for incorporation into polypeptide chains)</td>
<td>Anti-synthetase syndrome</td>
</tr>
<tr>
<td>Anti-Jo-1</td>
<td>Histidyl</td>
<td>Myositis, mechanic’s hands,</td>
</tr>
<tr>
<td>Anti-PL-7</td>
<td>Threonyl</td>
<td>Gottron’s papules, arthritis,</td>
</tr>
<tr>
<td>Anti-PL-12</td>
<td>Alanyl</td>
<td>fever, Raynaud’s</td>
</tr>
<tr>
<td>Anti-EJ</td>
<td>Glycyl</td>
<td>phenomenon, high frequency</td>
</tr>
<tr>
<td>Anti-OJ</td>
<td>Isoleucyl</td>
<td>of interstitial pneumonia</td>
</tr>
<tr>
<td>Anti-KS</td>
<td>Asparaginyl</td>
<td></td>
</tr>
<tr>
<td>Anti-Ha</td>
<td>Tyrosyl</td>
<td></td>
</tr>
<tr>
<td>Anti-SRP</td>
<td>SRP – intracytoplasmic protein translocation from ribosome into endoplasmic reticulum (6 polypeptides and ribonucleoprotein 7SLRNA)</td>
<td>Acute onset necrotizing myopathy</td>
</tr>
<tr>
<td>Anti-Mi-2</td>
<td>Helicase nuclear protein - transcription, remodelling of nucleosomes</td>
<td>Hallmark cutaneous DM, milder muscle disease</td>
</tr>
<tr>
<td>Anti-CADM-140</td>
<td>Intracytoplasmic MDA5 – innate immune responses against viral infections</td>
<td>CADM Rapidly progressive interstitial pneumonia</td>
</tr>
</tbody>
</table>

ARS, aminoacyl-tRNA synthetases; SRP, signal recognition particle; NuRD, nucleosome remodelling histone deacetylase; MDA5, melanoma-differentiation associated gene 5; DM, dermatomyositis; CADM, clinically-amyopathic dermatomyositis.
Systemic sclerosis specific:
- Topo-I: Topoisomerase
- RNAP I-III: RNA polymerase I-III
- Centromere
- Th/To RNP

Myositis-associated (overlap with systemic sclerosis):
- U1RNP
- U3RNP
- PM-Scl
- Ku
Anti-synthetase syndrome

- Myositis
- Raynaud’s phenomenon
- Arthritis
- Mechanic’s hands
- Gottron’s lesions
- Fever
- Interstitial pneumonia
1C: Myositis-specific autoantibodies

- Anti-synthetase – anti-synthetase syndrome
- Anti-SRP – acute necrotising myopathy
- Anti-Mi-2 – classic DM with hallmark skin disease
- Anti-MDA5 – clinically-amyopathic DM with significant lung involvement
1.8.4.2 Autoantigens and disease mechanisms

Identification of autoimmune targets in IIM has led to further insights into pathogenesis. Perhaps the most striking feature is that groups of autoantigens with analogous cellular functions are associated with similar clinical subsets. For example, the target molecules in the anti-ARS autoantibody phenotype (anti-synthetase syndrome) and the anti-SRP autoantibody phenotype (severe necrotizing myopathy) are found in the cytoplasm with distinct functions involved in protein synthesis and translation. In contrast, the other myositis autoantigen targets are all nuclear proteins involved in gene transcription and DNA processing. Recent work has proposed the hypothesis that the dysregulated autoimmune response in myositis syndromes is driven by specific antigens in distinct tissue microenvironments. Two studies have demonstrated that certain autoantigens are enriched in lesional tissue involved in IIM (107, 108). Several myositis-specific and associated autoantigens were shown to be upregulated in myositis muscle in comparison to normal muscle especially in regenerating muscle cells. Of further interest was the observation that Mi-2 was preferentially expressed in DM muscle rather than PM muscle. The theory that distinct microenvironments may shape disease expression was emphasised with the finding that a novel conformation of Jo-1 following cleavage by granzyme B is enriched in the alveolar-epithelial layer of the lung in comparison to other tissues including muscle (108). This has lead to the suggestion that the initiating target tissue for the autoimmune response in the anti-Jo-1 syndrome is the lung with secondary attack to muscle. Further evidence implicating autoantigen driven responses has been suggested by an antigen-induced model of IIM following immunisation of congenic mice with murine Jo-1 protein (133). Antibody responses were species-specific and following immunisation mice developed the ASS clinical phenotype with muscle and lung inflammation. The potential role of other autoantigens driving autoimmunity in IIM has been highlighted by work on the chromatin remodeler Mi-2. Using a conditional transgenic mouse model, a major function of the Mi-2 protein has been elucidated (134). In particular, Mi-2 is essential for development and repair of the basal epidermis, this observation may give further insight into the pathogenic mechanisms of the anti-Mi-2 autoantibody clinical subset, of which the predominant clinical manifestation is skin disease. Further evidence to suggest a central pathogenic role of the autoantigen in IIM comes from the demonstration that certain autoantigenic ARS proteins, histidyl (Jo-1) and asparaginyl (KS) can induce leukocyte migration. In contrast, non-autoantigenic ARS (aspartyl-tRNA and lysyl-tRNA synthetases) were not chemotactic. The study proposed that autoantigenic ARS are over-expressed in damaged muscle.
cells; their pro-inflammatory properties promote the immune response and thus the development of myositis (135). See Section 1.6.5, Figure 2.

1.8.4.3 MSAs and histopathological associations
There is also evidence to suggest that there are histopathological associations with certain MSAs, which highlights the overlap between the broad IIM subtypes. For example, patients with ASS associated myositis have distinct histopathological features with prominent perimysial inflammation, connective tissue fragmentation and perifascicular myopathic changes, in contrast to endomysial inflammation (136). Also, typical histopathological changes have been described in muscle biopsies from patients with anti-SRP autoantibodies; sparse inflammatory cell infiltrate, prominent muscle fibre necrosis and endomysial fibrosis (123, 124).
1.8.5 A proposed model for autoimmunity in IIM

Figure 2: Initiation of autoimmunity in IIM

Environmental factors (e.g. viruses / uv light)

Based on their studies, Casciola-Rosen and Rosen have proposed a hypothesis that the dysregulated autoimmune response in myositis syndromes is driven by novel patterns of antigen expression or conformation in distinct tissue microenvironments (107, 108, 137, 138). It is likely that a specific stimulus, for example tissue injury secondary to an infective or a toxic agent leads to a perturbed pro-immune setting in a genetically susceptible individual. In IIM, there may be three or four key initiating microenvironments i.e. muscle, lung or perhaps even skin or tumour tissue. As a result, altered antigen expression and the presentation of novel or previously unrecognised epitopes to the immune system leads to a loss of tolerance and initiation of disease. Subsequent generation of autoantibodies and generation of T cell responses may promote or propagate disease mechanisms.
1.9 Aims of study

As outlined in this chapter, in view of the clinical heterogeneity of these conditions diagnosing patients as purely DM or PM is perhaps too broad. Many investigators have proposed new sets of criteria based on updated insights. Studies suggest that clinical stratification is more appropriate according to the MSA / MAA, as the detected autoantibody and clinical phenotype are closely associated (6, 139, 140).

The hypothesis for this body of research is that adult and juvenile IIM is associated with autoantibodies that recognise specific autoantigen systems, which define patients into homogeneous clinical phenotypes. To address this hypothesis the overall aim of this thesis is to describe a comprehensive clinical and serological study of adult and juvenile IIM, including the identification of novel autoantibody subsets.

**Aim 1:**
To establish the prevalence and clinical features, in particular subtypes of recognised MSAs in the Royal National Hospital for Rheumatic Diseases (RNHRD) adult IIM cohort in comparison to previous published series.

**Aim 2:**
To investigate and characterise novel autoantibody specificities and corresponding autoantigen targets in patients recruited to the RNHRD and UK Adult Onset Myositis Immunogenetic Collaboration (AOMIC) IIM cohorts.

**Aim 3:**
To perform a clinical and serological study of children with JDM and JDM-overlap recruited to the UK and Ireland JDM Registry (UK JDM Cohort Study). To identify novel autoantibody-autoantigen systems, define clinical manifestations and thus markers of disease severity.
CHAPTER TWO
PATIENTS AND METHODS

2.1 Patients and sera
Subjects for the studies described in this thesis were recruited from three sources:

a) The Royal National Hospital for Rheumatic Diseases Adult Connective Tissue Disease Clinic (RNHRD)
b) The UK Adult Onset Myositis Immunogenetic Collaboration
c) The Juvenile Dermatomyositis Registry and Repository, UK and Ireland

2.1.1 Clinical diagnosis
Adult IIM patients, aged 18 years of age or older at disease onset, were recruited through the RNHRD and the UK Adult Onset Myositis Immunogenetic Collaboration, (AOMIC) (9). JDM patients were recruited to the UK and Ireland JDM National Registry and Repository (JDRR) (24). The JDRR has recruited patients with juvenile-onset myositis, below the age of 16 years at disease onset and diagnosis. All adult PM/DM and JDM cases had probable or definite disease according to Bohan and Peter criteria (1, 2). For adult myositis/CTD-overlap cases recruited to AOMIC, patients were included if they fulfilled all of the following: a) met published criteria for their primary CTD or mixed connective tissue disease (MCTD) (11, 12, 141, 142); b) possessed at least two of four Bohan and Peter criteria; (c) possessed at least one MSA/MAA. JDM-scleroderma overlap (JDM-SSc) was defined as JDM children with a history of Raynaud's phenomenon, sclerodactyly and other sclerodermatous skin changes (two or more of the above features).

Demographic and clinical data were recorded at diagnosis using standardised proformas (see Section 2.1.2 and 2.1.3). Serial clinical data was also available on patients attending the RNHRD and recruited through the JDRR. Data were stored using anonymous codes onto a central database. Serum and DNA samples were taken at the time of diagnosis and stored at -20°C until required.

2.1.2 Adult-onset inflammatory myopathy clinical proformas
See Chapter 2.5, Appendix I-III, pages 50-52.

2.1.3 Juvenile-onset inflammatory myopathy clinical proformas
See Chapter 2.5, Appendix IV, pages 53-60.
2.1.4 Preparation and storage of sera
Serum samples: approximately 5 ml of blood was venesected into a standard clotted sample tube. Once the blood had clotted, tubes were centrifuged at 3000 rpm for 10 min (Hereaus Labofuge 6000 Centrifuge). The serum fraction was extracted with a pipette into 500µl aliquots (adult samples) or 150µl aliquots (juvenile samples). Serum samples were stored at -20ºC.

2.1.5 Ethical approval
Collection of data and blood from patients and controls was undertaken under the regulation of the local research ethics committees and informed consent was obtained according to the Declaration of Helsinki.

2.2 Tissue culture techniques
All techniques were performed in class II tissue culture cabinets with sterile equipment. All reagents and media were purchased from Sigma, UK unless otherwise stated.

2.2.1 Preparation of tissue culture media

RPMI media
One litre of RPMI-1640 medium (with NaHCO₃, without L-glutamine) was supplemented with 10 ml 200 mM L-glutamine solution. Additionally, 100 ml foetal calf serum (heat inactivated) was also added for RPMI + FCS (10% foetal calf serum).

Preparation of dialysed foetal calf serum (FCS)
Two sections of dialysis tubing were cut (1 metre each, medium width), placed in a beaker of tap water and left under a running tap for 3-4 hr. The tubing was transferred to 300 ml 3% (v/w) sodium sulphide at 80ºC for 1 min, 300 ml distilled water at 60ºC for 2 min and 300 ml 0.2% (v/v) sulphuric acid at room temperature for 1 min before being rinsed in distilled water. Tubing was finally placed in 200 ml 0.02% (w/v) sodium azide and stored at 4ºC.

500 ml FCS was thawed at 37ºC overnight and PBS (2 x 3 l) was cooled at 4ºC overnight. The following day, lengths of treated dialysis tubing were rinsed in distilled water. FCS was transferred to the treated dialysis tubing, placed in 3 l cold PBS (prepared according to the manufacturer’s instructions) and stirred at 4ºC for 6 hr. The PBS was replaced with a further 3 l cooled PBS and stirred at 4ºC for a
further 22 hr. Dialysed FCS was transferred to a tissue culture flask and sterile filtered through a 0.22µM filter unit. Filtered FCS was aliquoted into 50 ml tubes and stored at -20°C until required. The sterility of the dialysed FCS was tested by adding 2 ml to 20ml RPMI + FCS and incubating at 37°C for 48 hr.

**Methionine free RPMI media (with dialysed foetal calf serum)**

One hundred ml RPMI-1640 medium (modified) (with NaHCO₃, without methionine, cystine and L-glutamine) was supplemented with 62 µl 10% (w/v) cystine (prepared in 2M HCl and sterile filtered) and 1 ml 200mM L-Glutamine. Five ml dialysed FCS was added for methionine deficient RPMI + 5% dialysed FCS.

### 2.2.2 Cell culture

The non-adherent K562 cell line (human chronic myelogenous leukaemic cells) was obtained from European Collection of Cell Cultures (Salisbury, UK) (89121407). Cells were stored under liquid nitrogen at the University of Bath and transported to the cell culture facilities on dry ice.

**Thawing K562 cell lines**

Vials of K562 cells in 90% FCS and 10% DMSO (dimethyl sulfoxide) were transferred to dry ice and transported to the BIRD laboratory, RNHRD. Aliquots were thawed at 37°C and added to 50 ml conical tubes containing 45 ml pre-heated RPMI + 10% FCS. Cells were incubated at 37°C for 10 min, before being centrifuged at 2000 rpm for 5 min (Heraeus Labofuge 6000). Supernatants were discarded and the pellets were resuspended in 25 ml pre-heated RPMI + 10% FCS. Cells were transferred to 60 ml flasks containing pre-heated RPMI + 10% FCS.

K562 cells were cultured and maintained in approximately 50 ml RPMI + 10% FCS, in vented, canted, tissue culture flasks. Cells were incubated in a 5% carbon dioxide humidifier incubator set at 37 °C.

### 2.3 Serological Methods

Anti-nuclear autoantibody screening was performed by indirect immunofluorescence of NOVALite HEp-2 cells INOVA Diagnostics, UK). Autoantibody specificity was further characterised by immunoprecipitation of radiolabelled cell extracts. All reagents and media were purchased from Sigma, UK unless otherwise stated.
2.3.1 **Indirect immunofluorescence**
Serum was incubated against a fixed substrate (HEp-2 cells) to allow binding of autoantibodies to their respective antigen(s). A secondary goat polyclonal antibody conjugated to fluorescein was incubated against the substrate-antibody used at 1/150 dilution in the assay. Slides (binding site slides) were mounted and studied under a fluorescence microscope. The presence of ANA was recognised by particular patterns of fluorescence.

2.3.2 **Radio immunoprecipitation (IPP) of protein targets**
Serum was incubated with protein-A coated Sepharose beads to enable IgG present in the serum to bind to the protein-A via the Fc fragment. Beads were washed and supernatant removed (to remove any unbound material) and the beads incubated with radiolabelled K562 cell extract. Beads were washed further and the supernatant removed. Samples were re-suspended in Laemmli sample buffer. Finally, samples were boiled to remove and denature bound autoantigens from the Sepharose protein-A and radiolabelled proteins were separated by SDS-PAGE electrophoresis. Gels were analysed following autoradiography, protein targets were recognised by the characteristic gel fingerprint and the molecular weight of the band.

**Figure 3: Protein immunoprecipitation (IPP)**

- Immobilise immunoglobulins from serum samples using protein-A linked to sepharose beads
- Select autoantibody targets by adding a detergent extract of radiolabelled proteins
- Add denaturing agents and separate proteins according to size using polyacrylamide gel electrophoresis
- Visualise radiolabelled proteins by autoradiography
**Preparation of the of K562 cell extract with Methionine**

K562 culture supernatants from 6 x 150 ml flasks were transferred by pipette to 8 x 50 ml conical tubes. These were centrifuged at 2000 rpm for 5 min (Heraeus Labofuge 6000). Supernatants were sterilely removed and the pellets were pooled and re-suspended in 2 conical tubes containing 40 ml methionine free medium (without dFCS). Cells were centrifuged at 2000 rpm for 5 min and the supernatants sterilely removed. Pellets were re-suspended in 2 conical tubes each containing 40 ml methionine free medium (with FCS). A cell count (1/10 dilution) was completed on each tube. 120 x 10^6 cells were added to 4 x 120 ml flasks and the flasks were topped up to 120 ml with methionine free medium (with dFCS). Cells were incubated at 37°C for 40 min. Two hundred and fifty µl radioactive methionine (Perkin-Elmer, UK EasyTag L-35S-Methionine, 5mCi (185 MBq) Stabilised Aqueous Solution) was added to each flask. The flasks were gently mixed and left at 37°C overnight. Culture supernatants were poured into 8 x conical tubes and were centrifuged at 2000 rpm for 5 min. The supernatants were discarded and the pellets pooled in 1 x conical tube containing 40 ml ice-cold Tris Buffered Saline (TBS) (10 mM Tris-HCl pH 7.4, 150 mM NaCl). A cell count was completed prior to centrifugation at 2000 rpm for 5 min. Supernatant was discarded and the cells were re-suspended in ice-cold immunoprecipitation (IPP) buffer (10 mM Tris-HCl pH 8.0, 500 mM NaCl 0.1% v/v Igepal) at a concentration of 12 x 10^6 cells / ml. The extract was sonicated using a Soniprep 150 MSE sonicator over iced-water in 7.5 ml batches (amplitude 4.5, 1 min on, 20 sec off x 4 or until no viscous lumps remained). Sonicated extract was pooled into a fresh 50 ml conical tube and then aliquoted into 1.5 ml tubes. Extract was centrifuged at 120000 rpm for 20 min at 1°C (MSE Hawk 15/05). Supernatants were pooled, transferred to fresh tubes 1.5 ml tubes (1 ml aliquots); snap frozen in dry-ice and ethanol and stored at -80°C. Any radioactive contamination was cleaned up using lipsol and appropriate standard operating procedures.

**Preparation of protein-A Sepharose beads**

Protein-A Sepharose beads were washed and hydrated in 40 ml IPP buffer by rotation at room temperature for 30 min. Hydrated Sepharose beads were centrifuged at 1100 rpm for 1 min (Heraeus Labofuge 6000). Supernatant was discarded and the pelleted beads were re-washed in 40 ml IPP buffer. Samples were re-centrifuged at 1100 rpm for 1 min, the supernatant was discarded and beads were re-suspended in IPP buffer at a concentration of 2 mg / ml.
**Immobilisation of Antibodies**

For each sample, 10 µl patient sera was mixed with 2 mg hydrated, washed protein-A Sepharose beads in 500 µl IPP buffer at room temperature for 30 min with end-over-end rotation. Autoantibody-coated Sepharose beads were pelleted at 2000 rpm for 1 min (MSE Hawk 15/05). Supernatant was discarded and the beads re-suspended in 1 ml ice-cold IPP buffer. The beads were washed a further 3 times by pelleting at 2000 rpm for 1 min, removing the supernatant and re-suspending in 1 ml ice-cold IPP buffer. After pelleting a final time at 2000 rpm for 1 min, the supernatant was discarded and the beads were resuspended in 380 µl ice-cold IPP buffer.

**Immunoprecipitation of Antigen**

Radiolabelled K562 cell extract was thawed and 120 µl was added to each sample. Samples were mixed with end-over-end rotation at 4°C for 2 hr. Samples were centrifuged at 2000 rpm for 1 min and the supernatant discarded. Beads were washed 3 times in IPP (as outlined in the paragraph above) and once in 1 ml ice cold TBS. Beads were pelleted a final time at 2000 rpm for 1 min, the supernatant was discarded and beads were re-suspended in 50 µl SDS Laemmli sample buffer. Samples were vortexed briefly and stored at -80°C until required.

2.3.3 SDS polyacrylamide gel electrophoresis

**Assembly of apparatus**

The Maxi gel electrophoresis equipment (Hoefer, UK) was assembled according to manufacturer’s instructions. Glass plates were washed with water, dried and cleaned with methanol. One ml thick spacers separated Glass plates and 15 well combs were used.

**Preparation of gels**

A 10% resolving gel was used as the standard for this procedure (see Chapter 2, Appendix V for gel recipes). Resolving gel mixture was poured into the assembled gel apparatus up to 2 cm from the top of the glass plates. Water saturated butan-2-ol (approximately 300 µl) was layered onto the top of each gel. Gels were left for at least 15 min to polymerise prior to being rinsed with distilled water. The components for a 5% stacking gel were mixed and layered on top of the resolving gel to the top of the glass plates. Teeth combs were inserted to approximately 1 cm from the top of the resolving gel. The stacking gel was left to polymerise for approximately 15 min prior to the combs being removed and the wells rinsed with distilled water.
**Sample preparation**  
Immunoprecipitated samples were taken out of the -80°C freezer to thaw. Samples were placed into the heating block and boiled at 95°C for 4 min, removed and briefly vortexed. Samples were centrifuged at 2000 rpm for 1 min (MSE Hawk 15/05) to pellet the beads.

**Gel loading and electrophoresis**  
Wells were loaded with 25 µl supernatant per sample. On each gel, 1 well was reserved for 15 µl pre-stained molecular weight marker (Biorad, UK). When not all the wells were used samples were spaced evenly across the gel. Once loaded, the gel apparatus was fully assembled according manufacture’s instructions and transferred to the buffer chamber along with a cooling coil. Four L running buffer (25 mM Tris-base, pH7.4 190 mM Glycine and 0.1% w/v SDS) was added to the lower chamber and 500 ml running buffer was added to the upper chamber. A constant current of 60 mA was run until the dye from the sample buffer and markers had passed through the stacking gel layer (approximately 1 hr). The current was then increased to 120 mA run until the dye had passed through the resolving gel (approximately 3 hr).

**Gel fixing and drying**  
Gels were removed from the glass plates and the stack layers discarded. The resolving gels were soaked in 0.5M sodium salicylate (enhancer) for 15 min in shallow trays. Salicylate solution was discarded; gels were rinsed in distilled water and placed in gel fix solution (methanol:water:glacial acid (4.5 :4.5:1 ratio)) for 30 min on a rocking platform. The fixative was drained off and the gels transferred onto blotting paper. The gels and blotting paper were placed on a gel dryer (Rapidry gel dryer ATTA Electrophoresis) and covered with cling-film. Gels were dried at 70°C for 70 min.

**Autoradiography**  
Labelled proteins were analysed by autoradiography. In a dark room, dried gels were placed into X-ray cassettes and overlaid with Kodax Biomax film (mat side down). Cassettes were stored in a -80°C freezer for 1-4 weeks (time stored was dependant on the age of the radioactive preparation and the immunoreactivity of the autoantigen target). Development of films was undertaken manually in the dark room. Films were placed in 47 ml Kodax developer solution mixed with 460 ml distilled water for 2 min (until bands were exposed). Films were rinsed in distilled
water and placed into 47 ml Kodax fixer solution mixed with 460 ml distilled water for 5 min. Films were immersed in distilled water again and then left to dry at room temperature.

See Chapter 2, Appendix VI, Table 3: summary data of molecular weights / apparent molecular weight run on 10% SDS-PAGE of recognised myositis-specific autoantigen targets.

2.3.4 Immunodepletion

See 2.3.2 for basic IPP methods and reagents. This technique was used to establish whether autoantibodies that recognised a novel autoantigen of the same molecular weight (in specific patient cohorts) were targeting the same protein specificity. In summary, radiolabelled cell extracts were immunoprecipitated with prototype sera (sera known to contain the novel autoantibody specificity): Serum was incubated with protein-A bound Sepharose beads and incubated with radiolabelled K562 cell extract. On this occasion, the pelleted beads bound to the autoantibody-autoantigen complex were removed and the supernatant (unbound cell extract) preserved. This procedure was repeated to ensure complete depletion of the target antigen from the cell extracts. Reference depleted cell extracts were then used for further immunoprecipitation with other autoantibody positive sera.

In detail, for each sample, duplicate tubes each containing 10 mg hydrated protein-A Sepharose beads (prepared as Section 2.3.2) and 50 µl reference sera in 1 ml IPP buffer were mixed with end-over-end rotation at room temperature for 30 min. Autoantibody-coated Sepharose beads were washed 4 x in IPP buffer (see section 2.3.2). After the beads were pelleted for the final time, supernatants were discarded. One tube for each sample (A) was placed on ice, whilst 150 µl [35S]-methionine-labelled K562 cell extract and 380 µl IPP buffer was added to the remaining tube (B). Tube B was mixed with end-over-end rotation at 4°C for 2 hr after which the supernatant was transferred to tube A. This was mixed at 4°C for a further 2 hr. The supernatant from tube A was transferred to a fresh tube (C) and stored at -80°C. Immunoprecipitations using positive sera and either 120µl control [35S]-methionine-labelled cell extract or the immunodepleted supernatants (C) were completed and subject to SDS-PAGE electrophoresis as described in Section 2.3.2.
2.3.5 Immunoprecipitation Western Blotting

Immunoprecipitation Western Blotting (IPP-WB) was used to determine the identity of specific autoantigens recognised by autoantibodies. Autoantigens were selected from patient's sera by immunoprecipitation and SDS-PAGE. Proteins were transferred to nitrocellulose membrane by immunoblotting. Membranes were incubated in a protein rich solution to block any remaining non-specific binding sites and probed with commercial antibody to a specific protein. Membranes were incubated with an appropriate secondary labelled anti-immunoglobulin followed by a conjugated staining substrate.

Preparation of unlabelled K562 cell extract

K562 cultures were grown to confluence in 6 flasks each containing 150 ml complete RPMI with FCS (see section 2.2.1). Supernatants were transferred to 8 x 50 ml conical tubes and centrifuged at 2000 rpm for 5 min (Heraeus Labofuge 6000). Supernatants were removed and the pellets resuspended in 2 x conical tubes each containing 40 ml ice-cold TBS. A cell count (1/10 dilution) was completed on each tube. Cells were centrifuged at 2000 rpm for 5 min, the supernatants were discarded and the pellets combined in a single conical tube containing ice-cold IPP buffer at a concentration of 15 x 10⁶ cells / ml. Extract was sonicated using a Soniprep 150 MSE sonicator over iced-water in 7.5 ml batches (amplitude 4.5, 1 min on, 20 sec off x 4 or until no viscous lumps remained). Sonicated extract was pooled into a fresh 50 ml conical tube and aliquoted into 1.5 ml tubes. Extract was centrifuged at 120000 rpm for 20 min at 1°C (MSE Hawk 15/05). Supernatants were pooled, transferred to fresh 1.5 ml tubes (1.2 ml aliquots); snap frozen in dry-ice and ethanol and stored at -80°C.

Antigen preparation for IPP WB

See 2.3.2 for IPP methods and reagents. Samples, each containing 40 μl serum and 2 mg hydrated protein-A Sepharose beads (prepared as Section 2.3.2) in 500 ml IPP buffer were mixed with end-over-end rotation at room temperature for 30 min. Autoantibody-coated Sepharose beads were centrifuged at 2000 rpm for 1 min (MSE Hawk 15/05), supernatant was discarded and beads re-suspended in 1 ml 0.2M tri-ethanolamine pH 8.1. Beads were washed a second time (centrifuged at 2000 for 1 min and re-suspended in tri-ethanolamine) prior to being centrifuged again and re-suspended in 5 mM bis-(sulphosuccinimidyl)-suberate cross-linker (Perbio, UK) in 1 ml 0.2M tri-ethanolamine. Beads were mixed with end-over-end rotation at room temperature for 30 min and centrifuged at 2000 rpm for 1 min.
Beads were re-suspended in 1 ml 50 mM Tris-Cl pH 7.5 and mixed with end-over-end rotation at room temperature for 15 min. Beads were centrifuged at 2000 rpm for 1 min and washed three times in PBS and two times in ice-cold IPP buffer. Beads were incubated in 1 ml unlabelled K562 cell extract with end-over-end rotation for 1 hr at 4°C, centrifuged at 2000 rpm for 1 min and resuspended in 1 ml fresh K562 unlabelled cell extract. Samples were incubated for a further 1 hr at 4°C with end-over-end rotation. Beads were washed 4 times in ice-cold IPP buffer and once in TBS. Samples were finally re-suspended in 80 µl SDS sample buffer and stored at -80°C until required.

**SDS-PAGE and transfer**

Proteins were fractionated using by 10% SDS-PAGE as per standard methods (see 2.3.3), however samples were loaded at 80 µl per lane. During the electrophoresis, blotting sheets and nitrocellulose membranes were soaked in Transfer Buffer pH7.4 (40mM tris, 20 mM Sodium acetate, 2mM EDTA, 20% v/v methanol and 0.1% w/v SDS). Once electrophoresis had been completed, gels were placed in the immunoblotting transfer apparatus according to manufacturers’ instructions. ‘Transfer sandwiches’ were prepared as scotchbrite, blotting paper, nitrocellulose membrane, gel, blotting paper and scotchbrite. The surfaces of the ‘transfer sandwiches’ were smoothed over to remove any air-bubbles. The ‘transfer sandwiches’ were placed into the tank apparatus that was filled with Transfer Buffer. A cooler system was inserted and proteins were transferred from the gels to the nitrocellulose membranes using a constant current at 220 mA for 4.5 hr. Nitrocellulose sheets were removed and the corners were marked. Membranes were rinsed in distilled water and were Ponceau stained for 5 min. Membranes were rinsed again for 4 x 15 sec in distilled water and air-dried overnight. Membranes were stored in a sealed container at 4°C until required.

** Immunoblotting and staining**

Membranes were washed in PBS-0.05%Tween for 5 min and blocked for 90 min in block solution (5% milk powder in PBS-0.05%Tween, pH7.4). Membranes were incubated with commercial primary antibody (diluted in block solution accordingly to manufacturer’s instructions) for 90 min. Membranes were washed 5 x 5 min in PBS-0.05%Tween and re-incubated with the corresponding secondary antibody (commercial anti-IgG) at a 1:20000 dilution for 90 min. Membranes were washed 5 x 5 min in PBS-0.05%Tween and finally incubated in substrate stock for 10-20 min. Membranes were rinsed with distilled water for 5 min and air-dried overnight.
2.4 Statistical analysis
Clinical and autoantibody associations were derived from 2×2 contingency tables using the chi-squared test with Yates’ continuity correction, or two-tailed Fisher’s exact test where individual cells valued five or less. Where significant, data were expressed as odds ratios (OR) with exact 95% confidence intervals (CI). Where data was not normally distributed the Mann-Whitney-U test was used to compare continuous data. Median values (inter-quartile ranges) were expressed where appropriate. \( P \) values \( (P_{\text{corr}}) \) were adjusted using the Bonferroni correction when comparing clinical associations. Uncorrected probabilities are presented for possible clinico-serological associations. \( P \) values <0.05 were considered significant. SPSS for Windows (version 14) was used to perform statistical analysis.
2.5 Appendix

I. RNHRD Clinical proforma (Page 50)

II. AOMIC Clinical Proforma (Page 51)

III. Autocure Clinical Proforma (Page 52)

IV. JDRR clinical Proforma (forms 1 and 2) (Pages 53-60)

V. Reagents and gel recipes (Pages 61-63)

VI. Table 3: Summary data of molecular weights / apparent molecular weight run on 10% SDS-PAGE of recognised myositis-specific autoantigen targets (Page 64)

Permission to reproduce AOMIC, Autocure and JDRR proformas granted by:
*Dr. Hector Chinoy, Consultant Senior Lecturer, University of Manchester
**Prof. Lucy Wedderburn, Scientific Director, JDRR, University College London
NOVEL AUTOANTIBODIES IN MYOSITIS STUDY

PATIENT DETAILS:

Name: Hospital no:

Sex: Ethnic origin:

D.O.B.: Age at disease onset:

CLINICAL DETAILS:

1. Presentation
   - Cutaneous disease only [ ]
   - Muscle disease only [ ]
   - Both [ ]

2. Development of myositis* in relation to cutaneous features (please tick):
   - Never [ ]
   - 3 months [ ]
   - 6 months [ ]
   - 12 months [ ]
   - Other – please specify [ ] months

3. Creatinine kinase at disease onset (if available) [ ]

4. Maximum creatinine kinase during follow up [ ]

5. Skin lesions (please tick):
   - Heliotrope rash [ ]
   - Gottron’s sign [ ]
   - Extensor aspect of fingers [ ]
     - Over elbows or knee [ ]
   - Periungual / nailfold erythema [ ]
   - V-sign rash [ ]
   - Shawl-sign rash [ ]
   - Mechanic’s hands [ ]
   - Calciosis [ ]

6. Other features (please tick):
   - Pyrexia [ ]
   - Weight loss [ ]
   - Dysphagia [ ]
   - Raynaud’s phenomenon [ ]
   - Arthritis [ ]
   - Cancer [ ]
   - Interstitial lung disease [ ]
     - Subtype (if known) – NSIP [ ]
       - UIP [ ]
       - Organising pneumonia [ ]

7. Other information e.g. type of cancer, extent of calciosis, treatment:

   [ ]

* myositis weakness / myopathic EMG / myositis on imaging or biopsy
### MYOSITIS GENETIC STUDY
#### CLINICAL & LABORATORY PROFORMA

**PATIENT DETAILS (complete or ring)**

**NAME:**

.................................................................................................................................

**Sex:** M / F  
**Hospital No.:** ......................................................

**Ethnic origin:**  
- Caucasian  
- Negroid  
- Asian  
- Oriental  
- Mixed (specify: .....................................................)  
- Other (specify: .....................................................)

**Current age:** ..............................................................

**Age at myositis onset:** .................................................

### MYOSITIS DIAGNOSTIC GROUP (tick or ring)

<table>
<thead>
<tr>
<th>Polymyositis (PM)</th>
<th>[ ]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dermatomyositis (DM)</td>
<td>[ ]</td>
</tr>
<tr>
<td>PM as part of other CTD</td>
<td>[ ]</td>
</tr>
</tbody>
</table>

(Specify: SLE / SSc / MCTD / Overlap / Undifferentiated)

### SYMPTOM / LESION INDEX

We will only include *definite PM/DM* according to the diagnostic criteria highlighted, so please tick which abnormalities are present:

1. **Skin Lesions**  
   - Heliotrope rash [ ]  
   - Gottron's sign [ ]  
   - Violeose rash on elbows/knees [ ]

2. **Systemic inflammatory signs** [ ]

3. **Non-destructive arthropathy** [ ]

4. **Muscle-specific antibody** [ ]

5. **Elevated skeletal muscle enzymes** [ ]

6. **Mylagia at rest or on contraction** [ ]

7. **Proximal muscle weakness** [ ]

8. **Myopathic EMG** [ ]

9. **Myositis on biopsy** [ ]

If patient has lung fibrosis as part of their PM, tick below.  
Lung fibrosis (clinically and/or on FFBs) [ ]

### DIAGNOSTIC CRITERIA FOR PM/DM

1. **Skin lesions:**  
   - Heliotrope rash (violaceous erythema on upper eyelids),  
   - Gottron's sign (violaceous keratotic papules on extensor aspects of fingers),  
   - Violaceous rash over elbows or knees.

2. **Systemic inflammatory signs** (elevated ESR/CRP, pyrexia, weight loss etc)

3. **Non-destructive arthritis**

4. **Muscle specific antibodies, e.g. Anti Jo-1 etc**

5. **Elevated skeletal muscle enzymes**

6. **Mylagia at rest or on contraction**

7. **Proximal muscle weakness**

8. **Myopathic EMG**

9. **Myositis on muscle biopsy.**

**For diagnosis of definite PM:**  
At least four from item list 2 – 9.

**For diagnosis of definite DM:**  
At least one feature from item 1), plus at least four from item list 2 – 9.

*(From Taniwato et al. Classification criteria for PM/DM. *J. Rheumatol* 1995, 22: 668-674)*

### UNDERLYING MALIGNANCY

If your patient has PM/DM, in association with a proven malignancy, could you please specify site & tissue type if known:

..................................................................................................................................................

..................................................................................................................................................

**Patient's Consultant:** ...........................................................................................................

**Hospital:** ............................................................................................................................

**Date form completed:** ........................................................................................................

**Signature:** ..........................................................................................................................

RGC/2000

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### AUTOCURE MYOSITIS

**CLINICAL & LABORATORY PROFORMA**

1. **CENTRE & PATIENT DETAILS**
   (complete / attach sticker):
   - **HOSPITAL** (Name/Code): ............................................................
   - **PATIENT (Name):** .................................................................
   - **Gender (m/f):** .................................................................
   - **NHS/Hospital No.:** ............................................................
   - **Ethnicity:** Caucasian/ Afro-Carib/ African/ Asian/ Oriental
     Mixed (specify) .................................................................
     Other (specify) .................................................................
   - **Date of birth:** .................................................................
   - **Month/Year of myositis onset:** ...........................................
   - **Month/Year of myositis diagnosis:** ....................................
   - **Height (cm):** .................................................................
   - **Weight (kg):** .................................................................
   - **Ever smoked?:** YES / NO

2. **MYOSITIS DIAGNOSTIC GROUP**
   (please tick one of a-d):
   - a) Polymyositis (PM) ............................................................
   - b) Dermatomyositis (DM) .......................................................
   - c) Amyopathic Dermatomyositis (ADM) ...................................
   - d) Inclusion Body Myositis
     (please complete IBM specific form) (IBM) ..........................
   - **ADDITIONALLY, if overlap with CTD:**
     - SLE/ Sjogren's/ MCTD/ SSc/ RA (ring)
     - Other (specify) .................................................................

3. **CASE VERIFICATION**
   (please tick all that apply):
   - 1. Proximal muscle weakness ..............................................
   - 2. Myositis on biopsy ...........................................................
   - 3. Elevated skeletal muscle enzymes ....................................
   - 4. Myopathic EMG ..............................................................
   - 5. Characteristic skin lesions ..............................................

4. **OTHER CLINICAL SIGNS**
   (please tick):
   - Heliotrope rash .................................................................
   - Gottron's papules .............................................................
   - Violaceous rash on elbows/knees ........................................
   - V sign .................................................................
   - Shawl sign .................................................................
   - Periungual erythema ........................................................
   - Mechanic's hands ............................................................
   - Raynaud's .................................................................
   - Arthritis .................................................................
   - Dysphagia .................................................................
   - Arrhythmias .................................................................

**REFERENCES:**


**DATE FORM COMPLETED**

---

**STUDY ID:**

---

**APPLICATIONS:**

- **5. AUTO-ANTIBODY DETECTION**
  - Myositis specific/associated autoantibody: YES / NO
  - Specify .................................................................
  - Non myositis-specific antibodies: YES / NO
  - Specify .................................................................

- **6. INTERSTITIAL LUNG DISEASE**
  - (attributable to myositis): YES / NO
  - Proven by (x-ray): PFTs / CXR / HRCT

- **7. MALIGNANCY:** (regardless of whether you consider the myositis to be cancer-associated)
  - Month/year of cancer diagnosis: ........................................
  - Site/Tissue type: ..........................................................

- **8. ENVIRONMENTAL FACTORS:**
  - (please ring/complete)
  - ? Statin / fibrates prior to onset of myositis: YES / NO
  - (specify drug if known): ................................................
  - Patient's view of what triggered myositis
  - (specify) .................................................................
  - Exposures: Asbestos/Silica/Fibreglass/Solvents/Coal dust
    - Other (specify) ..........................................................

**Patient's Physician:**

---

**REFERENCES:**

A. DEMOGRAPHICS:

DATE OF VISIT:  

PATIENT REF NO:

Patient status*: Male/Female

DOB:

Ethnicity: 01 02 03 04 05 06 07 08 09

(Please see guidelines for codes. If 04 or 09, please specify)

Postcode:

Occupation of parents:

*1=Initial visit/diagnosis at GOS/MDX
2=GOS/MDX primary institution for JDM care after previous diagnosis elsewhere
3=Seen for occasional care/shared care only (i.e. GOS/MDX not primary centre)
4=Other (please specify)

B. HISTORY UP TO DIAGNOSIS:

1. Background data

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<thead>
<tr>
<th>General symptoms</th>
<th>Absent</th>
<th>Present</th>
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<tr>
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<tr>
<td>Weakness</td>
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<tr>
<td>Fever</td>
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<td>1</td>
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<tr>
<td>Alopecia</td>
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<td>Weight loss</td>
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<tr>
<td>Fatigue</td>
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<td>Mouth ulcers</td>
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<td>Headache</td>
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<table>
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<td>Joint pain</td>
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<td>Jt stiffness</td>
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<td>Jt swelling</td>
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<td>Dyspnea</td>
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<td>Dysphonia</td>
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<td>Dysphagia</td>
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At diagnosis if available:

Height: _______cm
Weight: _______kg
BP: _______ / _______

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<td>Haematuria</td>
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<tr>
<td>Facial/body swelling</td>
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### 3. Past medical history

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<td>Other autoimmune disease</td>
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<td>Other significant diagnosis</td>
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<td>Trauma/injury</td>
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<td>GI problems</td>
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<td>Neurological involvement</td>
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<tr>
<td>FH of autoimmune disease</td>
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### C. EXAMINATION FINDINGS AT DIAGNOSIS:

#### I. Skin:

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<th>Condition</th>
<th>Absent</th>
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<tbody>
<tr>
<td>Gottron's papules</td>
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<td>Ulceration</td>
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<td>Oedema</td>
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<tr>
<td>Calcinosis</td>
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<td>Nailfold changes</td>
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<tr>
<td>Lipoatrophy</td>
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</table>

#### II. Arthritis

<table>
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<tr>
<th>Condition</th>
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<tbody>
<tr>
<td>Arthritis</td>
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#### III. Muscle weakness

<table>
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<tr>
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</thead>
<tbody>
<tr>
<td>Weakness</td>
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<td>1</td>
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</tbody>
</table>

#### IV. Other:

Specify _______

### D. CLINICAL COURSE SINCE DIAGNOSIS:

#### I. General symptoms:

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Absent</th>
<th>Present</th>
<th>Not known</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rash</td>
<td>0</td>
<td>1</td>
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<tr>
<td>Weakness</td>
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<td>Alopecia</td>
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<td>Weight loss</td>
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<td>Fatigue</td>
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<td>Mouth ulcers</td>
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<td>Headache</td>
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<td>Irritability</td>
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#### II. Musculoskeletal:

<table>
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<tr>
<th>Symptom</th>
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<tbody>
<tr>
<td>Myalgia</td>
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<td>Joint pain</td>
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<td>Jt stiffness</td>
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<td>Jt swelling</td>
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#### III. Systemic features:

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<td>Facial/body swelling</td>
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<tr>
<td>Calcinosis</td>
<td>0</td>
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</table>
### E. INVESTIGATION RESULTS AT DIAGNOSIS:

0 = normal, 1 = abnormal, 9 = not done. Please tick the appropriate box for each test result.

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F. THERAPY INFORMATION SINCE DIAGNOSIS

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<table>
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<td>Folic acid</td>
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G. PHYSIOTHERAPY/OCCUPATIONAL THERAPY

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<td>Hydrotherapy</td>
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<tr>
<td>Splinting</td>
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H. OTHER INFORMATION

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Specify:

Signature: ____________________________ Position: ____________________________
Print name: ____________________________ Date: ____________________________
# JUVENILE DERMATOMYOSITIS COHORT BIOMARKER STUDY AND REPOSITORY

## FORM 2: CLINIC VISIT FORM

**Patient Reference No:**  
**Date of visit:**

### A. HISTORY SINCE LAST CLINIC VISIT  
(or in last 3 months - whichever is most recent)

#### 1. Clinical History

<table>
<thead>
<tr>
<th>I. General Symptoms</th>
<th>Absent</th>
<th>Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rash</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Weakness</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Fever</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Alopecia</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Weight loss</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Fatigue</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Mouth ulcers</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Headache</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Irritability</td>
<td>0</td>
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<tr>
<td>Raynauds</td>
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<table>
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<td>Joint Pain</td>
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</tr>
<tr>
<td>Joint stiffness</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Joint Swelling</td>
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<td>1</td>
</tr>
<tr>
<td>Dyspnnea</td>
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<tr>
<td>Dysphonia</td>
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<tr>
<td>Dysphagia</td>
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#### III. Systemic Features

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<thead>
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<th>Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chest pain</td>
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</tr>
<tr>
<td>Abdo pain</td>
<td>0</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>0</td>
</tr>
<tr>
<td>Melena</td>
<td>0</td>
</tr>
<tr>
<td>Haematuria</td>
<td>0</td>
</tr>
<tr>
<td>Facial/body swelling</td>
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</tbody>
</table>

#### 2. Measurements

- Current height: ________ cm
- Current weight: ________ Kg
- BP: ________

#### 3. Family History

<table>
<thead>
<tr>
<th>No</th>
<th>Yes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
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</tbody>
</table>

Any new family history in the last 3 months: 0 1  
Specify:

---

## B. EXAMINATION FINDINGS

### 1. Skin

<table>
<thead>
<tr>
<th>Absent</th>
<th>Present</th>
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</thead>
<tbody>
<tr>
<td>Gottrons papules</td>
<td>0</td>
</tr>
<tr>
<td>Ulceration</td>
<td>0</td>
</tr>
<tr>
<td>Lipatrophy</td>
<td>0</td>
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<tr>
<td>Oedema</td>
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<tr>
<td>Nailfold changes</td>
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<tr>
<td>Calciosis</td>
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<tr>
<td>Other</td>
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</table>

Specify/describe: ____________________________________________

### II. Distribution of rash

<table>
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</thead>
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<tr>
<td>Periorbital</td>
<td>0</td>
</tr>
<tr>
<td>Periungal</td>
<td>0</td>
</tr>
<tr>
<td>Trunk</td>
<td>0</td>
</tr>
<tr>
<td>Small joints</td>
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<tr>
<td>Large joints</td>
<td>0</td>
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<tr>
<td>Other</td>
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Specify: ______________________
### III. Joints

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<th>Specify which joints are affected</th>
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<tr>
<td>Arthritis</td>
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<tr>
<td>Pain on motion</td>
<td>0</td>
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<td></td>
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<tr>
<td>Joints with limited ROM</td>
<td>0</td>
<td>1</td>
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<tr>
<td>Contractures</td>
<td>0</td>
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</tbody>
</table>

*0=no muscle action, 1=flicker of muscle action, 2=full action with gravity counterbalance, 3=muscle action against gravity, 4=muscle action against gravity with some resistance, 5=full muscle strength, (9=not done)*

### IV. Manual Muscle Testing

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<th>Muscle Group</th>
<th>Absent</th>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>9</th>
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<tbody>
<tr>
<td>Neck flexors</td>
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<td>3</td>
<td>4</td>
<td>5</td>
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<tr>
<td>Shoulder abductors</td>
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<td>2</td>
<td>3</td>
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<td>Elbow flexors</td>
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<tr>
<td>Wrist Extensors</td>
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<td>3</td>
<td>4</td>
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<tr>
<td>Hip Extensors</td>
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<td>3</td>
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### V. Oedema

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<td>Limb</td>
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<td>Trunk</td>
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### VI. Abdomen

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<td>Abdominal masses</td>
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<td>Tenderness</td>
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### VII. Respiration

Respiration: Please circle one: 0 | 1 | 2 | 3 | 4 | 5

0=normal, 1=SOBE, 2=tachypnoea, 3=accessory muscle use, 4=requires oxygen, 5=ventilated

### VIII. Other

<table>
<thead>
<tr>
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<th>Specify</th>
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### IX. Physician Global Assessment

**Disease least active:**

**Disease most active:**

8

### X. Physiotherapy/Occupational Therapy

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<tr>
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<td>CHAQ done today</td>
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<tr>
<td>CHQ done today</td>
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</tr>
<tr>
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<tr>
<td>Other</td>
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</tbody>
</table>
### D. INVESTIGATION RESULTS

0 = normal, 1 = abnormal, 9 = not done - tick the appropriate box for each result

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<th>VALUE</th>
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### F. THERAPY INFORMATION (since last visit)

#### I. Corticosteroids

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#### II. DMARDs

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#### III. NSAIDs

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Specify: _______________________

#### IV. Other Drugs

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<td>Gastroprotectant</td>
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<td>Vasodilator</td>
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Specify: _______________________

### F. RESEARCH INVESTIGATION SAMPLES

Were the following samples taken today:  

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<td>Clotted bottle</td>
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### G. OTHER INFORMATION

Specify: ______________________

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Signature: _______________________

Position: _______________________

Print Name: _______________________

Date: ______________________
V. **Reagents**

- RPMI x1 liquid (Sigma)
- 10% FCS or FBS (Sigma)
- L-glutamine, 200mM (Sigma)
- Methionine free medium; solid (Sigma)
- L-leucine; solid (Sigma)
- L-lysine; solid (Sigma)
- Sodium bicarbonate (Tissue culture grade, Sigma)
- S^{35} methionine (Amersham Biosciences (GE Healthcare) AG 1095 5mCi)
- Protein A or protein G sepharose beads (Sigma)
- Immunoprecipitation (IPP) buffer
  (10mM Tris/HCl, pH 8.0, 500mM NaCl, 0.1% v/v Igepal; previously known as Nonidet P-40)
- Tris buffered saline (TBS) buffer
  (10mM Tris/HCl, pH 7.4 + 150mM NaCl)
- Sodium Phosphate (crosslinking for MS)
  0.1M Sodium Phosphate pH8.1 (0.1M NaH_{2}PO_{4} to 500ml 0.1M Na_{2}HPO_{4} until pH=8.1)
- Tris-Cl (crosslinking stop buffer)
  1.0M Tris base pH7.5
- Triethanolamine (crosslinking reaction buffer)
  0.2M Triethanolamine pH8.1
- Laemmli sample Buffer (SDS-PAGE)
  Sigma S3401
- Western Blot Substrate
  Sigma B1911
- SDS-PAGE Marker
  Biorad Kaleidoscope Marker - 161-0324
- Blocking powder (Western Blot)
  5% Sainsburys semi-skimmed milk powder, pH 7.4 (with NaOH)
- PBS-Tween
  PBS with 0.05% Tween
- Phosphate Buffered Saline
  Sigma P4417 (1 tablet per 200 ml water)
- Western Blot Transfer Buffer
  40 mM Tris, 20 mM Sodium Acetate and 2 mM EDTA
  pH 7.4 with acetic acid
  Trizma base (Sigma)
V. Reagents

- Glycine
- Sodium chloride (BDH)
- Conc. HCl (Sigma)
- Glacial acetic acid (Sigma)
- PBS tablets (Sigma)
- Acrylamide/bis-acrylamide, 30% solution (Sigma)
- Ammonium persulphate (Sigma)
- Kalidescope markers, broad range containing glycerol, protein and bromophenol blue (Bio-Rad)
- TEMED (Sigma)
- Sodium dodecyl sulphate (SDS), (Sigma)
- Sample buffer containing mercaptoethanol, glycerol, bromophenol blue, tris and SDS (Sigma)
- Methanol (Sigma)
- Butan-2-ol (Sigma)
- Sodium salicylate (Sigma)
- DMSO (Sigma)
- Trypan blue (Sigma)
- Conc. Sulphuric acid (BDH)
- Sodium sulphide (Sigma)
- Sodium azide (Sigma)
- pH buffer standards (Lab 3)
### V. Gel recipes

Percentage acrylamide in gel mixture (volumes are per gel)

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<th>Vol (ml)</th>
<th>5%</th>
<th>8%</th>
<th>9%</th>
<th>10%</th>
<th>12.5%</th>
<th>15%</th>
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<td>Water</td>
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<td>Lower gel buffer</td>
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<tr>
<td>Upper gel buffer</td>
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<td>Acrylamide mixture</td>
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<td>9</td>
<td>10</td>
<td>12.5</td>
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30% (w/v) acrylamide:bisacrylamide (30:5:1)
Lower gel buffer stock: 1.5M Tris/HCl, pH 8.8 + 0.4% (w/v) SDS
Upper gel buffer stock: (*5% upper gels in this procedure) 0.5M Tris/HCl pH 6.8 + 0.4% (w/v) SDS
10% (w/v) ammonium persulphate
**VI. Table 3: Summary data of molecular weights / apparent molecular weight run on 10% SDS-PAGE of recognised myositis-specific autoantigen targets**

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<th>Autoantigen</th>
<th>Molecular Weight (kDa)</th>
<th>Runs at (10% SDS-PAGE) (kDa)</th>
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<td>PL-7</td>
<td>Threonyl-tRNA synthetase</td>
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<td>Alanyl-tRNA synthetase</td>
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<td>EJ</td>
<td>Glycyl-tRNA synthetase</td>
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<td>OJ</td>
<td>Multi-enzyme complex</td>
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<td>Leucyl-tRNA synthetase</td>
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<td>Tyrosyl-tRNA synthetase</td>
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<td>SRP</td>
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<td>Mi-2</td>
<td>SNF2 super-family helicase</td>
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CHAPTER THREE
RESULTS
RNHRD clinico-serological study of myositis-specific autoantibodies in adult-onset inflammatory myositis

3.1 Introduction
There is increasing evidence that patients with adult-onset idiopathic inflammatory myopathies (IIM); polymyositis (PM) and dermatomyositis (DM) have serological profiles within the disease spectrum (7, 46, 112, 139). Myositis specific autoantibodies (MSAs) appear to be important prognostic markers and are associated with specific clinical features.

The aim of this study, described in this chapter, was to investigate the prevalence of recognised MSAs in the Royal National Hospital for Rheumatic Diseases (RNHRD) IIM cohort in comparison to previous published series (6, 46, 112). This study also highlights the clinical features associated with the different MSAs, in particular lung disease. The work described here has been presented at the British Society of Rheumatology 2008 and the American College of Rheumatology 2006:


3.2 Patients and Methods
3.2.1 Patients
Patients with a diagnosis of adult IIM (age of onset 18 years or over) were identified from the RNHRD CTD database and clinics. Cases of myositis / connective tissue disease (CTD) overlap were excluded. The clinical notes were reviewed to confirm the diagnosis of PM or DM based on the Bohan and Peter criteria (1, 2), and clinical data were retrospectively recorded using a standardised proforma (see Chapter 2, Appendix 1). Data recorded included details on initial presentation, creatinine kinase (CK) at disease onset and maximum CK during disease course. The degree of
cutaneous involvement and other features were noted including the presence of lung disease and cancer-associated myositis.

3.2.2 Assessment of interstitial pneumonia
High resolution computerised tomography (HRCT) scans were re-reviewed under the guidance of Consultant Thoracic Radiologist (GR) and the subtype of interstitial pneumonia was categorised based on the American Thoracic Society / European Respiratory Society classification (32).

3.2.3 Serological Methods

ANA-Immunofluorescence (IIF)
IIF was previously performed by standard methods (PO and JD) using HEp-2 cells and fluorescein-labelled anti-human IgG immunoglobulin (Sigma, UK). The ANA IIF results were reviewed from the clinical notes and RNHRD / Bath Institute for Rheumatic Diseases serological database. The ANA titre and pattern was recorded.

Protein Radio-immunoprecipitation (IPP)
Sera stored at -20°C were thawed at room temperature. IPP from K562 cell extracts was performed as previously described in Chapter 2, see Sections 2.3.2 and 2.3.3. Briefly, 10 µl sera was mixed with 2 mg protein-A-Sepharose beads (Sigma, UK) in IPP buffer (10 mM Tris-Cl pH 8.0, 500 mM NaCl, 0.1% v/v Igepal) at room temperature for 30 min. Beads were washed in IPP buffer prior to the addition of 120 µl [³⁵S] methionine labelled K562 cell extract. Samples were mixed at 4°C for 2 hr. Beads were washed in IPP buffer followed by TBS buffer (10 mM Tris-Cl pH 7.4, 150 mM NaCl) before being resuspended in 50 µl SDS sample buffer (Sigma, UK). After heating, proteins were fractionated by 10% SDS-PAGE, enhanced, fixed and dried. Labelled proteins were analysed by autoradiography.

3.2.4 Ethical approval
All patients gave fully informed written consent to participate and provide biological samples according to the Declaration of Helsinki under local ethical committee regulations.

3.3 Results
Fifty patients with adult-onset IIM were identified from the RNHRD CTD database. The serological profiles and clinical associations are described below:
3.3.1 Serological profiles

Following IPP, autoantibody specificity was categorised into recognised MSAs, myositis-associated autoantibodies (MAAs), unidentified or negative. The frequency of recognised MSAs in this cohort of 50 patients was: anti-aminocyl-tRNA synthetases (anti-ARS) 28%, anti-SRP 8% and anti-Mi-2 4%. Fourteen% of cases had MAAs (8% anti-PM-Scl, 4% anti-Ro, 2% anti-Ku). Twenty-two% had autoantibodies that appeared to recognise distinctive autoantigen bands (see Chapter 4). Twenty-four% of patients sera were either negative or precipitated non-specific bands by IPP. See Table 4 for summary and Figure 4.

3.3.2 Clinical associations

The main clinical features of patients with MSAs; anti-ARS, anti-SRP, anti-Mi-2 and MAAs (anti-PMScl, anti-Ku, anti-Ro) are described in Table 5. The clinical associations of the novel autoantibody specificities are described in Chapter 4. The main clinical features of anti-ARS positive patients (n=14) were arthritis 78.6%, mechanic’s hands 57.1%, interstitial pneumonia 71.4% and Raynaud’s phenomenon 71.4%, and 71.4% initially presented with arthritis or respiratory disease prior to myositis onset. Anti-SRP positive patients (n=4) were characterised by severe disease; including severe myopathy (median CK 11036 IU/L), with systemic features of weight loss (50%) or fever (50%), arthritis (75%) and Raynaud’s phenomenon (75%) and cardiac involvement (50%). No anti-SRP patients had interstitial pneumonia. Muscle biopsies were available from two anti-SRP-positive patients demonstrating predominant muscle fibre necrosis, endomysial fibrosis and scanty inflammatory cell infiltrate. Both anti-Mi-2 positive cases had classic DM skin lesions and myositis but no other systemic organ involvement.

3.3.3 Subtypes of interstitial pneumonia

Ten (71.4%) anti-ARS-positive patients and two (29%) of MAA-positive patients had evidence of interstitial pneumonia on HRCT. No anti-Mi-2-positive, anti-SRP-positive or autoantibody-negative patients had evidence of interstitial pneumonia. As observed in previous studies (28-30, 47, 48), anti-ARS autoantibodies were a significant risk factor for interstitial pneumonia in comparison to other autoantibody subsets (anti-ARS-negative patients) ($P_{uncorr}=0.02$, odds ratio 2.4, 95% confidence interval 1.3-4.6). The interstitial pneumonia radiological subtypes are described in Table 6.
The main subtype of interstitial pneumonia observed in anti-ARS-positive cases was fibrotic non-specific interstitial pneumonia (NSIP), in 5 out of 10 patients. This HRCT appearance was defined by the degree of reticular changes, with predominately a subpleural and basal distribution. The fibrosing pattern was homogenous with absence of dense fibrosis i.e. no significant traction bronchiectasis or honeycombing. Three anti-ARS-positive patients (two anti-PL-7 and one with anti-PL-12 autoantibodies) had signs consistent with usual interstitial pneumonia (UIP) on HRCT, characterised by significant reticular changes including traction bronchiectasis and honeycombing. Four patients (two anti-Jo-1-positive and two anti-PM-Scl-positive cases) had HRCT features of limited NSIP i.e. predominately ground glass pattern, which is suggestive of fine fibrosis or alveolitis with a limited subpleural or basal distribution. One patient with limited NSIP had co-existent features of organising pneumonia (OP), characterised by patchy air-space consolidation, in the same distribution. See Figures 5-7 - HRCT images of corresponding interstitial pneumonia subtypes.
<table>
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<th>Autoantibody</th>
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<tr>
<td>Anti-PL-7</td>
<td>4</td>
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<tr>
<td>Anti-OJ</td>
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<tr>
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<td>&lt;5</td>
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<td>&lt;1</td>
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<td>5</td>
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<td>Anti-Mi-2</td>
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<td>Anti-Ro</td>
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<td>Anti-Ku</td>
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*Novel autoantibodies: anti-Zo, -p155/140, -p40/90 – see Chapter 4, p140 – see Chapter 7

**References: (112, 143)
Table 5: Clinical characteristics of anti-ARS, anti-SRP, anti-Mi-2 and MAA-positive patients

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<thead>
<tr>
<th>Autoantibody</th>
<th>Anti-ARS (n=14)</th>
<th>Anti-SRP (n=4)</th>
<th>Anti-Mi-2 (n=2)</th>
<th>MAAs (n=7)</th>
<th>Negative* (n=12)</th>
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<td>50</td>
<td>100</td>
<td>71</td>
<td>100</td>
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<td>Median age at onset (yrs)</td>
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<td>48.5</td>
<td>62.5</td>
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<td>11036</td>
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<td>33</td>
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*Negative or non-specific unidentified bands on IPP
Percentages are shown unless otherwise stated
Figure 4: Immunoprecipitation of myositis-specific autoantigens
Autoradiogram of 10% SDS PAGE of immunoprecipitates of $^{35}$S labelled K562 cell extracts. The reference sera used for immunoprecipitation were; Lane 1: normal serum (QC4), Lane 2: anti-Jo-1 (QC78), Lane 3: anti-PL-12 (R12501), Lane 4: anti-PL-7 (R18134), Lane 5: anti-EJ (AOMIC MM182), Lane 6: anti-KS (AOMIC MM020), Lane 7: anti-OJ (AOMIC MM63), Lane 8: anti-Zo (R21845), Lane 9: anti-SRP (R7417), Lane 10: anti-Mi-2 (R18411).

Arrows indicate molecular weights (kDa) –see Chapter 2, Appendix, Table 4 for running molecular weights of 10% SDS-PAGE.
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</tr>
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<td>Anti-Jo-1</td>
<td>Limited NSIP</td>
</tr>
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<td>Fibrotic NSIP</td>
</tr>
<tr>
<td>R12707</td>
<td>Anti-Jo-1</td>
<td>Limited NSIP and OP</td>
</tr>
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<td>Anti-PL-7</td>
<td>UIP</td>
</tr>
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<td>UIP</td>
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<td>Fibrotic NSIP</td>
</tr>
<tr>
<td>R21845</td>
<td>Anti-Zo*</td>
<td>Fibrotic NSIP</td>
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<td>R8125</td>
<td>Anti-PM-Scl</td>
<td>Limited NSIP</td>
</tr>
<tr>
<td>R10686</td>
<td>Anti-PM-Scl</td>
<td>Limited NSIP</td>
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</tbody>
</table>

*See Chapter 4*
Figure 5: Non-specific interstitial pneumonia

Figure 6: Usual interstitial pneumonia

Figure 7: Organising pneumonia
3.4 Discussion

Historically, PM and DM has been defined based on broad pathological and clinical criteria (1, 2). There is now increasing evidence that these disorders characterised by a wide clinical spectrum can now be classified into more homogeneous clinico-serological syndromes (6, 46, 112, 139). Autoantibodies found exclusively in IIM, termed MSAs are directed against cytoplasmic or nuclear components involved in key regulatory processes such as protein synthesis, translocation and nuclear transcription, and have been the subject of major interest over the last few years. Unlike myositis-associated autoantibodies (MAAs) found in principally myositis-scleroderma overlap syndromes; MSAs are highly selective, mutually exclusive and are associated with characteristic clinical profiles. As described in Chapter 1; several myositis specific autoantigen systems have been described including the aminoacyl-tRNA synthetases (ARS) (111, 114-119), signal recognition particle (SRP) (121, 122) and Mi-2 (126).

Consistent with previous observations (112), in this study the most frequently detected MSA group were anti-ARS, with a lower frequency of anti-SRP and anti-Mi-2 autoantibodies. Using IPP, a number of novel autoantibody specificities were identified, which are described fully in Chapter 4.

The clinical manifestations of anti-ARS-positive patients were consistent with the anti-synthetase syndrome, with a high frequency of mechanic’s hands, fever, arthritis, Raynaud’s phenomenon and interstitial pneumonia. As previously described in anti-synthetase syndrome (42), the majority of patients presented with extra-muscular manifestations, in particular interstitial pneumonia, prior to their myositis onset. Clinically-amyopathic disease or milder muscle involvement is described especially in non-Jo-1 anti-ARS-positive cases (42, 144-148). In comparison to anti-ARS-negative cases, anti-ARS-positivity was a significant risk factor for the presence of interstitial pneumonia, as observed previously (6, 31, 42, 48, 144-147, 149-152). Consistent with previous findings (30, 38), NSIP was the frequent interstitial pneumonia subtype observed in anti-ARS-positive patients (including all anti-Jo-1-positives). Interestingly, anti-PL-7 or -12 positivity was associated with UIP, the radiological and histological pattern synonymous with idiopathic pulmonary fibrosis. Indeed, the predominant clinical manifestation in two of these patients was interstitial pneumonia, with one patient (anti-PL-7-positive) developing myositis approximately 10 years after the onset of lung disease and one...
patient (anti-PL-12-positive) has never developed clinical myositis. In some cases, IPF may actually represent a formes frustes of autoimmune connective tissue disease, especially in patients with non-Jo-1 anti-ARS (150), which may not be tested for in routine clinical practice. In a study by Matsushita et al 25% of patients with idiopathic interstitial pneumonia were anti-ARS positive (152). At present, it remains unclear why Jo-1 is the most common autoantigenic target in ASS or why patients with anti-ARS autoantibodies have differences in their clinical presentation. In these patients, a high index of clinical suspicion including the recognition of other subtle clinical features, for example a history of arthralgia or fever, is required.

Similar to previous studies (122-124), all patients with anti-SRP autoantibodies had evidence of severe myopathy with markedly elevated CK at disease onset. In addition, patients had systemic features, cardiac involvement and arthritis but no significant skin disease, and none had evidence of IP. Therefore, identification of anti-SRP autoantibodies at disease onset has prognostic implications, identifying those patients at risk of severe muscle disease that may be refractory to standard treatments and thus require more intensive management. Anti-Mi-2 is a DM-specific-autoantibody (6, 10, 125-128), and in this study, both patients with anti-Mi-2 autoantibodies had myositis associated with classic cutaneous DM lesions with no systemic features or internal organ involvement.

In summary, the MSAs: anti-ARS, -SRP, -Mi-2, identify IIM patients into three distinct clinical phenotypes. Rather than diagnose patients as purely DM or PM, if we adopt a serological classification to define patients into clinical syndromes this may help predict outcomes, in particular, identify those patients who are at risk of severe disease, which in turn may influence treatment strategies.
## 3.5 Appendix

### I. SUBJECTS

<table>
<thead>
<tr>
<th>Code</th>
<th>IIF pattern</th>
<th>IPP result</th>
<th>HRCT pattern</th>
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*See Chapter 4

N.A. – not applicable

ANA IIF patterns - abbreviations:

C: coarse
D: diffuse
F: fine
S: speckle
NS: nucleolar sparing
II. Acknowledgements

Mrs J Dunphy (JO) and Mrs P Owen (PO) previously performed ANA IIF on sera from IIM cases as part of the RNHRD (Bath Institute for Rheumatic Diseases) Diagnostic Laboratory Service. I would like to thank them for giving me permission to include this data in this study.

I would also like to acknowledge and thank Dr G Robinson (GR), Consultant Radiologist, Royal United Hospital, Bath for his supervision and teaching in the interpretation of HRCT scans.

I would like to thank all the patients who have attended the RNRHD CTD clinic and given their consent to participate in these studies.
CHAPTER FOUR
RESULTS
Identification and characterisation of novel autoantigen systems in adult-onset inflammatory myositis

4.1 Introduction
To date, several myositis specific autoantibodies (MSAs) have been described including the anti-aminoacyl-tRNA synthetases (ARS) (6, 114-119), -signal recognition particle (SRP) (6, 123, 124) and -Mi-2 (6, 125-127) (as described in Chapter 1, see Table 2, for summary). Over the last few years, new MSAs have been described in adult IIM.

Sato et al have described an autoantibody termed anti-CADM-140 in a Japanese cohort of adult patients with clinically amyopathic dermatomyositis (CADM) (130). As defined by Gerami et al, patients with CADM have hallmark cutaneous features of DM but no clinical evidence of myositis (40). Moreover, CADM patients especially those of Asian origin, appear to have an increased risk of more severe interstitial pneumonia (31, 41, 153). In this study by Sato et al, anti-CADM-140 autoantibodies were detected in 8 out of 42 DM patients (19%) but not in patients with PM, other CTDs or control groups. All 8 patients with anti-CADM-140 autoantibodies were clinically amyopathic. Anti-CADM-140-positive sera demonstrated a granular or reticular cytoplasmic staining pattern on immunofluorescence (IIF) using HEp-2 cells. Following radio-immunoprecipitation (IPP) using 35S-methionine-labelled K562 cell extract, anti-CADM serum recognised an ~140 kDa polypeptide, which was distinguishable from other known MSAs, and immunodepletion studies confirmed that this autoantibody recognises the same target. Of interest, the frequency of severe interstitial pneumonia was significantly increased in the anti-CADM-140-positive group compared to anti-CADM-140 negatives (50% versus 6%, p=0.008) (130). Using a series of molecular techniques including a HeLa cell-derived complementary DNA library, the identity of the ~140 kDa protein has recently been identified as a RNA helicase encoded by melanoma differentiation-associated gene 5 (MDA5) (131). MDA5 is involved in innate immune responses against viruses (132, 154, 155). This finding suggests that a specific viral infection in genetically susceptible individuals may play a direct role in the pathogenesis of CADM and severe interstitial pneumonia, particularly as this MSA has been detected in Japanese patients, and confirmed in a further cohort recently (156).
A further novel MSA, termed anti-p155 or anti-p155/140 has been reported in adult DM. In the study by Targoff et al, following IPP using HeLa cell extract, anti-p155-positive sera recognised a distinct 155 kDa protein (along with a weaker 140 kDa protein in most cases) in 8 out of 39 DM patients (21%). Anti-p155 sera revealed a nuclear speckled pattern on IIF with HEp-2 cells (143). Kaji et al have demonstrated what is likely to be the same autoantibody, termed anti-p155/140, in 7 out of 52 DM patients (13%) using IIP with K562 cells (157). Both studies showed that anti-p155/140 autoantibodies also appear to identify a distinct clinical phenotype with more severe skin involvement. Moreover, the most striking feature is the clear association with malignancy and Chinoy et al have confirmed this. The authors showed that anti-p155/140 positivity confers a significant risk for cancer-associated myositis with a high specificity, moderate sensitivity and high negative predictive value (158). In preliminary work, the identity of the p155 protein target is consistent with transcriptional intermediary factor 1-gamma (TIF-1-γ), a nuclear transcription protein involved in cellular differentiation (159).

This chapter describes data from the RNHRD IIM cohort on anti-p155/140 autoantibodies, and also work describing two novel MSAs (termed anti-Zo and anti- SAE). The work in this chapter has been published, as listed below:

4.2 Patients and Methods

4.2.1 Patients

Patients with a diagnosis of adult IIM (age of onset 18 years or over) were identified from the RNHRD CTD database and clinics. Cases of myositis / CTD overlap were excluded. The clinical notes were reviewed to confirm the diagnosis of PM or DM based on the Bohan and Peter criteria (1, 2), and clinical data were recorded using a standardised proforma (see Chapter 2, Appendix 1). Data recorded included details on initial presentation, creatinine kinase (CK) at disease onset and maximum CK during disease course. The degree of cutaneous involvement and other features were noted including the presence of lung disease and cancer-associated myositis. For the studies on anti-SAE autoantibodies (see Section 4.3.2) clinical data and serum samples were also available from the UK Adult Onset Myositis Immunogenetic Collaboration (AOMIC) (collaborators Dr Hector Chinoy and Dr Robert Cooper).

Sera from disease controls (150 with SSc / 40 with SLE) and 40 normal controls were also analysed (available from the RNHRD Bath Institute for Rheumatic Diseases Repository). All patients with SSc and SLE fulfilled recognised published criteria (11, 12).

4.2.2 Serological Methods

ANA Immunofluorescence (IIF)

IIF was previously performed by standard methods (PO and JD) using HEp-2 cells and fluorescein-labelled anti-human IgG immunoglobulin (Sigma, UK).

Protein Radio-immunoprecipitation (IPP)

Sera stored at -20°C were thawed at room temperature. IPP from K562 cell extracts was performed as described in Chapter 2, see Sections 2.3.2 and 2.3.3. Briefly, 10 µl sera was mixed with 2 mg protein-A-Sepharose beads (Sigma, UK) in IPP buffer (10 mM Tris-Cl pH 8.0, 500 mM NaCl, 0.1% v/v Igepal) at room temperature for 30 min. Beads were washed in IPP buffer prior to the addition of 120 µl [³⁵S] methionine labelled K562 cell extract. Samples were mixed at 4°C for 2 hr. Beads were washed in IPP buffer followed by TBS buffer (10 mM Tris-Cl pH 7.4, 150 mM NaCl) before being resuspended in 50 µl SDS sample buffer (Sigma, UK). After heating, proteins were fractionated by 10% SDS-PAGE, enhanced, fixed and dried. Labelled proteins were analysed by autoradiography.
**Immunodepletion experiments**

As described in Chapter 2, section 2.3.4: duplicate samples each containing 10 mg protein A Sepharose beads in 1 ml IPP buffer and 50 µl serum were mixed with end-over-end rotation at room temperature for 30 min. Beads were washed four times in 1 ml IPP buffer and 1 tube (A) was placed on ice whilst 150 µl [³⁵S] methionine-labelled K562 cell extract and 350 µl IPP buffer was added to the remaining tube (B). Tube B was mixed at 4°C for 2 hr after which the supernatant was transferred to tube A, this was mixed at 4°C for a further 2 hr. The supernatant from tube A was then transferred to a fresh tube (C) and stored at -80°C. IPP using reference sera and either 150 µl control [³⁵S] methionine-labelled cell extract or the immunodepleted supernatants (C) were completed as described for IPP using [³⁵S] methionine.

**Protein isolation and mass spectrometry**

Autoantigen isolation and identification experiments were carried out by ZB (see Appendix 4.5 for details). Therefore, the methodology has not been described in Chapter 2, but is outlined in brief in this section. Forty µl of reference sera was mixed with 2 mg protein-A-Sepharose beads in 500 µl IPP buffer at room temperature for 30 min with end-over-end rotation. Beads were washed two times in 1 ml 0.2M triethanolamine pH 8.2 (Sigma, UK) and bound antibodies were crosslinked to the beads using 5mM bis-(sulphosuccinimidyl)-suberate (Pverbio, UK) in 1 ml triethanolamine, mixing at room temperature for 30 min. The reaction was stopped with 1 ml 50mM Tris-Cl pH 7.5, mixing at room temperature for 15 min. The antibody coated Sepharose beads were washed three times in phosphate-buffered-saline and twice in IPP buffer prior to the addition of 1 ml K562 cell extract, corresponding to approximately 1 x 10⁶ cells. Samples were mixed end-over-end rotation at 4°C for 1 hr. The supernatant was removed and the beads were resuspended in a further 1 ml K562 cell extract and were mixed for 1 hr at 4°C. Beads were washed four times in IPP buffer and once in TBS before being resuspended in 80 µl SDS sample buffer. After heating (95°C for 4 min), proteins were fractionated by 10% SDS-PAGE. Gels were washed 3 x 5 min in pure water, stained for 60 min using Imperial Protein Stain (Pverbio, UK) and destained overnight in pure water. Unique bands were removed to a 96-well plate.

**Mass Spectrometry (MS)**

Samples were prepared for MS at the University of the West of England using an Ettan robotic digester (GE Biosciences). Gel pieces were destained in 50%
Methanol / 50mM Ammonium Bicarbonate, dehydrated in 70% acetonitrile, air dried and digested overnight at room temperature with 20 ng/µl modified porcine trypsin (Promega) in 20mM ammonium bicarbonate. Peptides were extracted from gel pieces to a clean plate using 50% Acetonitrile / 0.1% TFA (trifluoroacetic acid) that was then dried down. The peptides were redissolved in 50% Acetonitrile / 0.1% TFA and mixed with an equal amount of 10 mg/ml alpha-cyano-4-hydroxycinnamic acid before being spotted on the MALDI target plate using an Ettan Robotic Spotter (GE Biosciences, UK). Peptide mass fingerprints were acquired using Waters Micromass Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF) with data acquisition and processing carried out using the MassLynx software. The database searching was performed by ProteinLynx software.

4.2.3 Ethical approval
All patients gave fully informed written consent to participate and provide biological samples according to the Declaration of Helsinki under local ethical committee regulations.

4.3 Results
4.3.1 Anti-Zo, a novel autoantibody in the anti-synthetase syndrome

Identification of anti-Zo autoantibodies to phenylalanyl tRNA synthetase

Prior to this study, seven anti-aminoacyl-tRNA (ARS) synthetase autoantibodies had been described (see Chapter 1, Section 1.6.4.1 and Table 1). As part of the work related to the study described in Chapter 3, it was noted that two IIM sera (R15222 and R21845) from the RNHRD cohort study had a strong discrete cytoplasmic speckle on IIF using HEp-2 cells, the pattern of which was suggestive of anti-ARS autoantibodies (see Figure 8). Protein IPP with the two prototype sera produced a novel pattern with two bands at approximately 60 and 70 kDa (see Figure 9A). The molecular weights of the bands immunoprecipitated did not match any of the known tRNA synthetases associated with myositis, including Jo-1, PL-7 and PL-12 (see Figure 9B) and as described for KS, EJ and OJ (see Chapter 3, Figure 4), implying that both index cases serum (R15222 and R21845) IPP pattern was due to a novel anti-ARS autoantibody. This pattern was not observed in any other disease or control groups.

In order to further characterise the anti-ARS, the corresponding autoantigen was purified and identified using SDS-PAGE and MALDI-TOF MS. Sera from either the index cases or patients with known autoantibodies to Jo-1, PL-7 or PL-12 were used

83
to immunoprecipitate autoantigens from a K562 cell extract. A Coomassie stained SDS-PAGE of the immunoprecipitates showed bands of expected molecular weight for the Jo-1, PL-7 and PL-12 controls (see Figure 9B) as well as 60 and 70 kDa bands from the index case. The bands were digested by trypsin, analysed by MS and SwissProt database matched. Matches required peptide coverage of over 20% and scores of approximately 12. All matches were repeated on at least two separate occasions for the controls and three separate occasions for the index case immunoprecipitates. Table 7 demonstrates the correct identification of Jo-1, PL-7 and PL-12 autoantigens in the control sera and the database matching of the autoantigens precipitated by the index case. On each of the three separate occasions, the 60 kDa band was matched to phenylalanyl tRNA synthetase alpha chain (57 kDa protein) and the 70 kDa band was matched to phenylalanyl tRNA synthetase beta chain (66 kDa protein).

Clinical features of anti-Zo autoantibodies

Index case 1 (R15222)

A 49 year old woman presented with shortness of breath; six months later she developed proximal muscle weakness, Raynaud’s phenomenon, puffy fingers, mechanic’s hands and arthralgia. Clinical findings demonstrated a proximal myopathy with a creatinine kinase (CK) of 9533 IU/L. Muscle biopsy confirmed a necrotising myopathy with inflammatory cells. Pulmonary function tests revealed a restrictive pattern and high-resolution computerised tomography (HRCT) showed signs of fibrotic non-specific interstitial pneumonia (NSIP).

Index case 2 (R21845)

A 34 year old man presented with shortness of breath on exertion. Over the next three months he developed Gottron’s papules on the extensor aspects of his fingers, mechanic’s hands, proximal myopathy with a CK of 4276 IU/L (muscle biopsy demonstrated a necrotising myopathy with inflammatory cell infiltrate), fever, Raynaud’s and arthritis. HRCT thorax showed signs of fibrotic NSIP.

In summary, both patients with anti-Zo autoantibodies had typical clinical manifestations of the anti-synthetase syndrome.
Figure 8: ANA IIF – anti-Zo autoantibodies
Indirect immunofluorescence staining of Hep-2 cells after incubation with index case 1 serum (R15222) and fluorescein-labelled anti-human immunoglobulin. The cytoplasmic speckle seen at 40x magnification is shown.
Figure 9: IPP - anti-Zo autoantibodies
A – Autoradiogram of 10% SDS-PAGE of immunoprecipitates using [$^{35}$S] labelled K562 cell extracts. B – Coomassie stained 10% SDS-PAGE of immunoprecipitates using unlabelled K562 cell extract. Sera used for immunoprecipitation include; Lane 1; control serum; Lanes 2-5, anti-synthetase sera with autoantibodies to; Lane 2 – Jo-1; Lane 3 – PL-7; Lane 4 – PL-12 and Lane 5 – index case serum, R15222 (containing autoantibodies to phenylalanyl tRNA synthetase). Molecular weight markers are indicated on the left of panel B and bands corresponding to the tRNA synthetase targets are marked.
Table 7 - Results of Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) and SwissProt database matching. Comparison of peptide fragments of antigens precipitated by serum containing anti Jo-1, PL-7 and PL-12 along with peptides from the 60 kDa and 70 kDa bands precipitated by two index cases sera. Mass peptide fingerprints matched to histidyl-, threonyl- and alanyl-, phenylalanyl (alpha chain)- and phenylalanyl (beta chain)- tRNA synthetase respectively. Matches were deemed positive if the peptide coverage was over 20%, the same major theoretical and experimental peaks were present and the maximal MALDI-TOF score (using the software and database combination) was approximately 12.

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</table>

1Mwt – theoretical molecular weight, 2PI – theoretical isoelectric point.
4.3.2 Anti-SAE: autoantibodies targeting small ubiquitin-like modifier activating enzyme in adult dermatomyositis

4.3.2.1 Anti-SAE: autoantibodies targeting small ubiquitin-like modifier activating enzyme in adult dermatomyositis (RNHRD study)

Identification of anti-SAE autoantibodies in index cases
The RNHRD IIM study described in Chapter 3 identified two DM patients with the same previously unidentified autoantibody pattern (R16316 and R11040). Sera from these patients gave a speckled nucleolar sparing staining pattern on IIF (R16316 serum also showed a fine cytoplasmic speckle) (data not shown) and were found to immunoprecipitate proteins of approximately 40 kDa and 90 kDa (see Figure 10) that did not correspond to any known autoantigen profile. This pattern was not observed in any other disease or control groups.

Autoantigen identification
Immunodepletion studies were completed to demonstrate that the IPP pattern seen with R16316 and R11040 were due to the precipitation of the same autoantigens. Cell extracts were depleted of antigen targets using normal, R16316 and R11040 sera before being used in further IPP using R16316 and R11040 sera. When the cell extracts were pre-depleted with normal serum, the 40 and 90 kDa antigens were still visible after IPP with each of the patient’s sera (see Figure 10, Lanes 2 and 6). However, after pre-depletion with either patient’s sera and removal of the corresponding targets, the 40 and 90 kDa bands were absent after subsequent IPP by either patient’s sera (see Figure 10, Lanes 3-4 and 7-8). These results therefore provided good evidence that the serum from R16316 and R11040 both contained the same autoantibody specificity.

Identification of the SAE antigens
Non-radiolabelled IPP and Coomassie stained SDS-PAGE demonstrated the presence of 40 kDa and 90 kDa bands using both R16316 and R11040 serum (see Figure 11). MALDI-TOF MS and Swiss Prot analysis of the peptide fingerprints from these bands corresponded to SUMO 1 Activating Enzyme A subunit (SAE1) (38 kDa protein) for the 40 kDa band and SUMO 1 activating enzyme B subunit (SAE2) (71 kDa protein) for the 90 kDa band (see Table 8).
Clinical features of anti-SAE autoantibodies in index cases

Index Case 1 (R16316)
A 52 year old lady presented with a three month history of DM rash, including periungual erythema, V-sign rash and heliotrope rash. She had no muscle symptoms with a normal CK and electromyography (EMG). Six months later she developed proximal myopathy (CK 797 IU/L), dysphagia, and worsening skin disease with the Shawl-sign rash. Muscle biopsy confirmed necrotising myopathy with persistent inflammatory change. Screening investigations showed no sign of an underlying malignancy. HRCT showed limited peripheral lung involvement consistent with limited mild NSIP.

Index Case 2 (R11040)
A 62 year old man presented with clinical features of cutaneous DM, including a heliotrope rash and Gottron's papules. Six months later he developed proximal myositis with pharyngeal involvement. CK was 1086 IU/L and the diagnosis was confirmed following EMG study. Further investigations excluded an underlying malignancy but did show changes consistent with peripheral limited mild NSIP.

In summary, the results from this study suggest that anti-SAE autoantibodies may form a further serological subset in adult IIM with similar clinical manifestations. A further patient (R20894) was subsequently identified as anti-SAE-positive, with this finding included in the anti-SAE UK AOMIC study described below.
Table 8 – Results of MALDI-TOF MS and SwissProt database matching. Comparison of mass peptide fragments of antigens precipitated by patient 1 and 2 sera to small ubiquitin like 1 activating enzyme (SAE1) and (SAE2). Results from a total of three separate occasions (with two matches for R16316 and one match for R11040) are shown. Matches were deemed positive if the peptide coverage was over 20 percent and the same major theoretical and experimental peaks were present.

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</table>

1Mwt –theoretical molecular weight, 2PI – therotical isoelectric point.
4.3.2.2 Anti-SAE autoantibodies in UK AOMIC cohort

The AOMIC registry has recruited UK Caucasian patients with adult-onset myositis, 18 years or over at disease onset, from hospitals around the UK. All patients had probable or definite myositis according to the Bohan and Peter diagnostic criteria (1, 2). A standardised proforma, including demographic and clinical data, was used throughout (see Chapter 2, Appendix). Cancer associated myositis defined as cancer occurring within 3 years of diagnosing myositis (as per the modified Bohan and Peter classification used in a previous study (9)) was confirmed by the local clinician caring for individual patients. Patients were investigated for interstitial pneumonia based on clinical symptoms and pulmonary function testing and where indicated HRCT thorax. Clinical data and serum samples from 266 myositis patients were available for analysis for this study. The median age of disease onset was 50 years (IQR 37-61) and 195 (73%) cases were female. One hundred and thirty one cases had DM, 124 PM and 11 myositis / CTD overlap. Sera and DNA samples were taken at the time of diagnosis and stored at -20°C until required.

Identification of anti-SAE autoantibodies in UK AOMIC cohort

Sera from 266 myositis patients were screened for anti-SAE autoantibodies. Excluding the reference cases (R16316 and R11040) (as described in Section 4.3.2.1), nine further patients were identified as having 40 kDa and 90 kDa bands on SDS-PAGE, previously described as corresponding to SAE1 and SAE2 respectively (see Section 4.3.2.1 and Reference (160)). The presence of anti-SAE autoantibodies in these sera was confirmed by immunodepletion experiments. Immunoprecipitations on the 40kDa and 90 kDa positive sera were completed using cell extracts depleted with either the index case serum (anti-SAE), or with healthy control serum. All sera were positive for the 40 kDa and 90 kDa bands using healthy control depleted cell extract but were negative for the 40 kDa and 90 kDa bands with the index case (anti-SAE) depleted extract (see Figure 12). These results provide confirmation that the serum from the index cases and the nine additional patients contain the same autoantibody specificity, anti-SAE. Anti-SAE autoantibodies were not detected in any of the disease or normal control groups.

None of the 11 anti-SAE positive sera were found to co-immunoprecipitate any other recognised MSAs, although 5 patients did co-immunoprecipitate additional unidentified non-specific bands on IPP. IIF on anti-SAE positive sera demonstrated a fine speckled nucleolar-sparing pattern (titres ranging from 1/160 to 1/2560) in ten
patients with two sera also showing a fine cytoplasmic speckle (1/40 titre) and a
homogeneous pattern (1/640 titre) in one case.

Clinical features of anti-SAE autoantibodies in UK AOMIC cohort
The overall frequency of anti-SAE autoantibody positive cases in the myositis cohort
was 4%. Anti-SAE was found exclusively in DM patients (n=131), with a frequency
of 8%. The detailed clinical features of the 11 anti-SAE positive patients are
described in Table 9. Seven (64%) were female and the mean age was 62 years
(IQR 54-68). Nine patients (82%) had a heliotrope rash, 9 (82%) patients had
Gottron's lesions on the fingers and 7 (64%) had Gottron's over the large joints
(elbow or knee). Eight out of 8 patients (100%) (data not available on 3 patients)
had periungual changes. Seven out of 9 patients (78%) (data not available on 2
patients) had dysphagia, 3 of which had profound swallowing dysfunction, which
required enteral feeding. Nine out of 11 patients (82%) had systemic features as
defined by fever, weight loss and raised inflammatory markers. Two patients (18%)
had cancer-associated myositis. Two patients (18%) had radiological evidence of
ILD (limited non-specific interstitial pneumonia with less than 10% lung involvement
on HRCT scored by a thoracic radiologist) but neither of these 2 patients had
respiratory symptoms. Seven anti-SAE positive cases (78%) (data not available on 2
patients) presented with cutaneous DM only (no weakness and normal CK), but then
went onto develop frank myositis. The median disease duration between the initial
presentation of skin disease and myositis onset was 3 months (IQR 1, 6). After
multiple corrections were applied, no significant clinical associations were observed
when comparing anti-SAE positive patients with anti-SAE negative DM patients
except for a higher frequency of periungual changes in the anti-SAE positive group
($P_{corr}=0.03$). In addition, data on the type of presentation i.e. skin disease first or skin
/ muscle disease at onset was not available on the anti-SAE negative DM group to
make this comparison. The data is summarised in Table 10. This study has
confirmed that SAE is an important autoantigen target in DM with specific clinical
associations.
Table 9: Clinical features of patients with anti-SAE autoantibodies in UK AOMIC cohort

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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Raised CK</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>ILD</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>CAM</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Arthritis</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Presentation (months)@</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S/M</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>~</td>
<td>S/M</td>
<td>~</td>
</tr>
<tr>
<td>(months)@</td>
<td>(6)</td>
<td>(6)</td>
<td>(6)</td>
<td>(3)</td>
<td>(2)</td>
<td>(3)</td>
<td>(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Key to Table 9:
*Gottron’s lesions over the extensor aspects of fingers
**Gottron’s lesions over the extensor aspects of elbows or knees
~Clinical information not available
^Weakness – objective signs defined by manual muscle testing
MH (mechanic’s hands)
ILD (interstitial lung disease) – patients asymptomatic / limited mild non-specific interstitial pneumonia on high resolution CT scan
CAM (cancer-associated myositis)
@S=presented skin disease first (number of months before myositis onset), S / M = presented with skin and muscle disease
Cases 1 and 2 (index cases R16316 and R11040 – RNHRD study)
<table>
<thead>
<tr>
<th>Clinical Feature</th>
<th>Anti-SAE positive (n=11)**</th>
<th>Anti-SAE negative (n=120)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>64</td>
<td>74</td>
</tr>
<tr>
<td>Heliotrope</td>
<td>82</td>
<td>71</td>
</tr>
<tr>
<td>Gottron's papules (fingers)</td>
<td>82</td>
<td>63</td>
</tr>
<tr>
<td>Gottron's lesions (other)</td>
<td>64</td>
<td>48</td>
</tr>
<tr>
<td>Periungual erythema</td>
<td>100 #</td>
<td>46</td>
</tr>
<tr>
<td>V-sign rash</td>
<td>43</td>
<td>35</td>
</tr>
<tr>
<td>Shawl-sign rash</td>
<td>43</td>
<td>37</td>
</tr>
<tr>
<td>Dysphagia</td>
<td>78</td>
<td>43</td>
</tr>
<tr>
<td>Systemic</td>
<td>82</td>
<td>54</td>
</tr>
<tr>
<td>Weakness</td>
<td>100 #</td>
<td>89</td>
</tr>
<tr>
<td>Elevated CK</td>
<td>82</td>
<td>85</td>
</tr>
<tr>
<td>Interstitial pneumonia</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>CAM</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>Arthritis</td>
<td>18</td>
<td>22</td>
</tr>
</tbody>
</table>

*Values are the percentage of patients who had the clinical feature. **Not all patients had data on each clinical feature. CAM: cancer-associated myositis. #\( P_{corr} = 0.03 \) compared to anti-SAE negative DM patients.
Figure 10: IPP - anti-SAE autoantibodies

IPP of antigens recognised by patients R16316 and R11040. Autoradiogram of 10% SDS-PAGE of immunoprecipitates using either R16316 and R11040 serum with control $^{35}$S methionine-labelled cell extract or $^{35}$S methionine-labelled cell extract depleted with either normal sera (NS), patient R16316 sera (1) or patient R11040 sera (2). The bands corresponding to SAE1 (40 kDa band) and SAE2 (90 kDa band) are indicated.
Figure 11: Coomassie stained IPP of proteins using anti-SAE antibodies
Coomassie stained 10% SDS-PAGE of immunoprecipitates using either patient 1 (R16316) or 2 (R11040) crosslinked serum precipitated with unlabelled K562 cell extract. The molecular weight markers are indicated in kDa on the left and the bands corresponding to SAE1 (40 kDa band) and SAE2 (90 kDa band) are indicated on the right.
**Figure 12: SAE Immunodepletion**

Autoradiogram of 10% SDS-PAGE of immunoprecipitates using anti-SAE positive patient serum and either normal serum depleted $[^{35}\text{S}]$ methionine – labelled cell extract (A) or SAE depleted $[^{35}\text{S}]$ methionine – labelled cell extract (B) NS – IPP of normal serum and undepleted $[^{35}\text{S}]$ methionine – labelled cell extract, SAE – IPP using anti-SAE positive serum and undepleted 35S methionine. Bands corresponding to SAE1 (40 kDa band) and SAE2 (90 kDa band) are indicated.
4.3.3 Anti-p155/140: autoantibodies targeting 155/140 kDa doublet protein in adult dermatomyositis

Identification of anti-p155/140 autoantibodies

The RNHRD IIM study described in Chapter 3 identified seven DM patients with the same previously unidentified autoantibody pattern and similar clinical features:

<table>
<thead>
<tr>
<th>Patient</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>R7505</td>
<td>R13064</td>
</tr>
<tr>
<td>R8315</td>
<td>R16815</td>
</tr>
<tr>
<td>R9316</td>
<td>R 20125</td>
</tr>
<tr>
<td>R11248</td>
<td></td>
</tr>
</tbody>
</table>

Following IPP testing, sera from the above cases of adult DM recognised two distinct proteins forming a doublet with molecular weights of 155 kDa and 140 kDa (see Figure 13). In each case a weak, non-specific nuclear pattern was detected on IIF (data not shown).

Immunodepletion studies were undertaken in order to ascertain whether the IPP pattern seen in the adult DM cases was due to precipitation of the same autoantigens. Immunoprecipitations on the different anti-p155/140 kDa positive sera were completed using cell extracts depleted with a reference index case anti-p155/140 serum (R7505), or with healthy control serum. All sera were positive for the p155/140 kDa bands using healthy control depleted cell extract but were negative for the p15/140 kDa bands with the index case (R7505) depleted extract (see Figure 14). These results provide confirmation that the serum from the index case and the six additional patients contain the same autoantibody specificity, anti-p155/140. None of the seven anti-p155/140 positive sera were found to co-immunoprecipitate any other recognised MSAs.

Frequency of anti-p155/140 autoantibodies

A total of seven out of 50 (14%) adult IIM patients were positive for autoantibodies to the p155/140 kDa doublet. Anti-p155/140 was only detected in adult DM cases with a frequency of 26.9%. Anti-p155/140 autoantibodies were not found in any of the disease or normal control groups.

Clinical features of anti-p155/140-positive patients

Findings are summarised in Table 11 and Table 12. Four adult DM patients with anti-p155/140 had a history of malignancy, which was diagnosed either at onset or
within three months of their DM. The remaining three anti-p155/140 patients had clinically-amyopathic DM (CADM) based on clinical findings and normal creatinine kinase levels since diagnosis, and no history of malignancy (median seven years from diagnosis to time of study).

In comparison to anti-p155/140 negative adult DM patients, anti-p155/140-positive patients had a higher frequency of cutaneous lesions; including V-sign rash, Shawl sign rash, heliotrope rash and Gottron’s lesions. In addition, systemic manifestations including dysphagia were more frequent. No anti-p155/140-positive patients had interstitial pneumonia.

There was a significant association with anti-p155/140-positivity and malignancy ($P_{cor}=0.03$, odds ratio 7.3). The types of cancer seen in this group of patients were varied but all patients had evidence of solid organ malignancy.
Table 11: Clinical features of anti-p155/140-positive adult DM vs. anti-p155/140-negative adult DM *

<table>
<thead>
<tr>
<th>Feature</th>
<th>Anti-p155/140 Positive (n=7)</th>
<th>Anti-p155/140 Negative (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age at diagnosis (yrs)</td>
<td>54 (33, 71)</td>
<td>51 (49, 58)</td>
</tr>
<tr>
<td>Females</td>
<td>71.4</td>
<td>89.5</td>
</tr>
<tr>
<td>Heliotrope rash</td>
<td>85.7</td>
<td>63.2</td>
</tr>
<tr>
<td>Gottron's papules</td>
<td>100</td>
<td>84.2</td>
</tr>
<tr>
<td>Periungual erythema</td>
<td>71.4</td>
<td>63.2</td>
</tr>
<tr>
<td>V-sign rash</td>
<td>71.4</td>
<td>26.3</td>
</tr>
<tr>
<td>Shawl-sign rash</td>
<td>85.7</td>
<td>26.3</td>
</tr>
<tr>
<td>Dysphagia</td>
<td>57.1</td>
<td>21.0</td>
</tr>
<tr>
<td>Weight loss</td>
<td>28.6</td>
<td>26.3</td>
</tr>
<tr>
<td>Weakness</td>
<td>57.1</td>
<td>73.7</td>
</tr>
<tr>
<td>Elevated CK</td>
<td>57.1</td>
<td>78.9</td>
</tr>
<tr>
<td>Interstitial pneumonia</td>
<td>0</td>
<td>31.6</td>
</tr>
<tr>
<td>CAM</td>
<td>57.1 #</td>
<td>0</td>
</tr>
<tr>
<td>Arthritis</td>
<td>28.6</td>
<td>36.8</td>
</tr>
<tr>
<td>Calciosis</td>
<td>14.3</td>
<td>0</td>
</tr>
<tr>
<td>Mechanics hands</td>
<td>0</td>
<td>15.8</td>
</tr>
</tbody>
</table>

*Values are in percentages unless otherwise indicated

CK: creatinine kinase
CAM: cancer-associated myositis

#P corr=0.03 compared to anti-p155/140 negative DM patients (P=0.002), odds ratio 7.3, 95% CI 2.56-20.99)
<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Age at onset</th>
<th>Malignancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DM</td>
<td>82</td>
<td>Small cell carcinoma adrenal metastasis</td>
</tr>
<tr>
<td>2</td>
<td>DM</td>
<td>71</td>
<td>Bone and liver metastasis</td>
</tr>
<tr>
<td>3</td>
<td>DM</td>
<td>50</td>
<td>Breast carcinoma</td>
</tr>
<tr>
<td>4</td>
<td>DM</td>
<td>77</td>
<td>Gastric carcinoma</td>
</tr>
<tr>
<td>5</td>
<td>CADM</td>
<td>28</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>CADM</td>
<td>24</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>CADM</td>
<td>56</td>
<td>No</td>
</tr>
</tbody>
</table>

CADM: clinically-amyopathic dermatomyositis
Figure 13: IPP anti-p155/140 autoantibodies

Immunoprecipitation of p155/140 kDa autoantigens and other selected MSAs. 10% SDS PAGE of immunoprecipitates of [35S] labelled K562 cell extracts. Sera used for immunoprecipitation include Lanes 1-6; adult anti-p155/140 positive serum, Lane 7; threonyl tRNA synthetase, Lane 8; alanyl tRNA synthetase, Lane 9; histidyl tRNA synthetase, Lane 10; Mi-2, Lane 11; signal recognition particle and Lane 12; normal serum. Positions of the p155 and p140 antigens are indicated on the left. Positions of the Mi-2 bands at 240, 65 and 63 kDa are indicated. The 54, 83 and 108 kDa molecular weight markers correspond to signal recognition particle, threonyl tRNA synthetase and alanyl tRNA synthetase respectively.
Figure 14: Immunodepletion experiments (anti-p155/140)

Immunodepletion experiments - Autoradiogram of 10% SDS-PAGE of immunoprecipitates using adult positive anti-p155/140 serum: R7505, R8315 and R9316. Immunoprecipitation was performed with control $[^{35}S]$ methionine-labelled cell extract or $[^{35}S]$ methionine-labelled cell extract depleted with either normal serum (NS) or adult positive anti-p155/140 serum (R7505). The bands corresponding to the p155 and p140 autoantigens are indicated.

<table>
<thead>
<tr>
<th>Depletion serum</th>
<th>None</th>
<th>NS</th>
<th>R7505</th>
<th>R7505</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPP serum</td>
<td>R8315</td>
<td>R9316</td>
<td>R8315</td>
<td>R9316</td>
</tr>
</tbody>
</table>
4.4 Discussion
This chapter outlines work that characterises three new MSAs in adult IIM. The first is a new anti-ARS (-Zo) that targets phenylalanyl tRNA synthetase, which is the eighth autoantigenic ARS to be identified. The second is an autoantibody that targets SAE, an enzyme involved in post-translational modification, a completely novel system in adult DM. Finally, the clinical specificity of anti-p155/140 autoantibodies are described in patients recruited to the RNHRD IIM cohort study.

4.4.1 Anti-Zo; autoantibodies against phenylalanyl tRNA-synthetase
The aminoacyl tRNA-synthetases are a distinct group of enzymes that catalyse the ATP-dependent binding of an amino acid to its cognate tRNA during protein synthesis. ARS are functionally related enzymes; each amino acid has its own corresponding tRNA-synthetase, except glutamic acid and proline, which have both been shown to be catalysed by a single polypeptide encoding the ‘gluprolyl’ tRNA synthetase (163). Previously, seven anti-ARS have been described (see Chapters 1 and 3) (111, 114-119). ARS belong to one of two classes depending on the amino acid they are responsible for. Both classes are multi-domain proteins with a catalytic domain and an anti-codon binding domain (ensures binding of the correct tRNA to the protein). Five ARS; histidyl (Jo-1), threonyl (PL-7), alanyl (PL-12), asparaginyl (KS) and glycyl (EJ) are class II synthetases found free in the cell cytoplasm. They have three highly conserved motifs, are generally dimeric or tetrameric, and attach their amino acid to the 3’ hydroxy (OH) of their tRNA. The other two autoantigenic ARS: isoleucyl (OJ) and tyrosyl (Ha) are class I enzymes with two highly conserved motifs, are generally monomeric, and attach the carboxyl of their target amino acid to the 2’ OH of adenosine 76 in the tRNA molecule. Anti-OJ autoantibodies are primarily directed against isoleucyl-tRNA synthetase but can react against multiple synthetases as part of a class I multi-enzyme complex including leucyl and glutaminyl-tRNA (117). Prior to this study; several ARS have not be shown to be autoantigenic (see Table 13). Either these synthetases are not targeted as part of an autoimmune response or autoantibodies directed against them are extremely rare. It is also feasible that it has not been possible to identify autoantibodies to the remaining synthetases using conventional diagnostic techniques.

Phenylalanyl tRNA synthetase is the eighth autoantigen associated with anti-synthetase syndrome to be identified. Similar to the majority of autoantigenic ARS, phenylalanyl is a class II synthetase but it aminoacylates at the 2’ OH of the same adenosine, rather than at 3’ OH.
Manifestations of anti-synthetase syndrome are described in Chapters 1 and 3. It is noteworthy that the two index cases identified as having anti-Zo autoantibodies had clinical features consistent with this syndrome. In particular, as well as myositis, both patients developed NSIP, the presence of which influenced treatment. As highlighted in Chapters 1 and 3, a major and sometimes predominant manifestation of anti-synthetase syndrome is interstitial pneumonia, which can be the major factor in terms of prognosis (42, 48). The different manifestations are not universal between patients and it is possible that each anti-ARS may define more specific clinical subsets within the syndrome itself. This is perhaps most notable in patients with non-Jo-1-anti-ARS (-KS, -PL-12, -PL-7) with lung disease in the absence of clinically apparent myositis (42, 48, 118, 144, 145). In a study by Yoshifuji H et al, the frequency of interstitial lung involvement in their patients with anti-synthetase syndrome was as high as 95%. Interestingly in terms of interstitial pneumonia being the first disease manifestation; anti-ARS autoantibodies were detected in 77% of this subgroup of patients with lung disease preceeding myositis by a median of 11 months. However, in patients who developed myositis first, anti-ARS were only found in 20%. This study also looked at patients with interstitial pneumonia sine myositis; defined by anti-ARS, lung disease, no muscle disease either clinically or biochemically and no cutaneous IIM signs. All patients in this subgroup had non-Jo-1-anti-ARS (48). Similarly, the index anti-Zo-positive cases in this study initially presented with respiratory symptoms; the onset of myositis with other characteristic features occurring later. It is possible that non-Jo-1-anti-ARS may identify patients at one end of the spectrum. The question remains do anti-ARS have a predictive value in the context of idiopathic interstitial pneumonia i.e. do a proportion of these patients actually have a “forme fruste” of autoimmune CTD, as suggested in previous studies (48, 150). Detection of anti-ARS autoantibodies in these patients may influence treatment strategies, as highlighted by Yoshifuji H et al, who showed that patients with anti-ARS-associated interstitial pneumonia respond significantly better to corticosteroids compared to “seronegative” interstitial pneumonia. However the anti-ARS-positive group were also shown to have a higher rate of relapse with recurrent lung disease (48). This suggests the need to treat patients aggressively with early intervention, with slower tapering of corticosteroids together with additional immunomodulatory therapy. From a clinical perspective, early identification of this subset of patients may influence management. Furthermore, studies described in Section 1.6.4.2 suggest a potential direct role of both candidate autoantigens and specific anti-ARS autoantibodies in disease pathogenesis that may lead to more specific targeted therapies (80, 107, 135, 164).
Table 13: Aminoacyl-tRNA synthetases

<table>
<thead>
<tr>
<th>Class I tRNA synthetases</th>
<th>Class II tRNA synthetases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginyl</td>
<td>Alanyl (PL-12) †</td>
</tr>
<tr>
<td>Cysteinyl</td>
<td>Asparaginyl (KS) †</td>
</tr>
<tr>
<td>Glutamyl</td>
<td>Histidyl (Jo-1) †</td>
</tr>
<tr>
<td>Glutaminyl*</td>
<td>Glycyl (EJ) †</td>
</tr>
<tr>
<td>Isoleucyl (OJ)* ‡</td>
<td>Lysyl</td>
</tr>
<tr>
<td>Leucyl*</td>
<td>Phenylalanyl (Zo) †</td>
</tr>
<tr>
<td>Methionyl</td>
<td>Prolyl</td>
</tr>
<tr>
<td>Tryptophanyl</td>
<td>Seryl</td>
</tr>
<tr>
<td>Tyrosyl (Ha) ‡</td>
<td>Threonyl (PL-7) †</td>
</tr>
<tr>
<td>Valyl</td>
<td></td>
</tr>
</tbody>
</table>

*autoantigenic ARS molecules

*autoantibodies to isoleucyl (OJ) also react against other synthetases as part of a class I multi-enzyme complex including leucyl and glutaminyl-tRNA
4.4.2 Anti-SAE autoantibodies

Work described in this chapter has confirmed that SAE is an important autoantigen target in IIM where it is associated with specific clinical and associations. Anti-SAE, like anti-Mi-2, appears to be highly specific for DM. The frequency of anti-SAE in the AOMIC / RNHRD DM cohort was 8%. This is in comparison to Mi-2 which, depending on detection methods, is seen in 10-20% of DM patients (126, 128). Anti-SAE autoantibody positive DM patients have signs of hallmark cutaneous disease whereas interstitial lung involvement appears to be rare, and when present is mild. Systemic features including weight loss and dysphagia are a common manifestation of this subgroup. Furthermore, anti-SAE may be an important prognostic marker in patients who initially present with cutaneous DM. The majority of anti-SAE positive cases presented with skin lesions first and all went onto develop myositis. This finding requires further validation in larger patient groups such as those presenting with clinically-amypathic DM under the care of dermatologists. The association with cancer and anti-SAE does not appear as strong as the now well recognised association with anti-p155/140 autoantibodies as described previously (143, 157, 158), and also in this chapter. Interestingly, the frequency anti-SAE autoantibodies with cancer-associated myositis were similar to that found for anti-Mi-2 autoantibodies in a recent study (158). In the future, larger studies will facilitate more detailed investigation of the clinical specificity of anti-SAE in comparison to other DM-specific autoantibodies.

Post-translational modifications are essential for full biological activity and allow a rapid cellular mechanism in response to changes in the environment. However, these modifications can also lead to the creation of novel self-antigens and the development of autoimmunity. Examples of this can be seen with glycosylation and hydroxylation modifications in collagen-induced arthritis, citrullination in rheumatoid arthritis and phosphorylation in SLE (165).

Small ubiquitin-like modifiers (SUMO) play a key role in the post-translational modification of specific proteins. The SUMO family consists of four members of which SUMO-1 is the best characterised. Sumoylation leads to the formation of stable conjugates of target proteins including protein kinases and transcription factors (166-169). Studies have implied a role for sumoylation in nucleo-cytoplasmic transport and signal transduction (167, 168). SUMO has been linked to certain inflammatory diseases. Franz et al demonstrated high levels of SUMO-1 mRNA expression in rheumatoid synovial tissue compared to controls. The relevance of
this over-expression is unclear but it was suggested that through inhibition of apoptosis, the survival of synovial fibroblasts may be increased (170). In addition, Janka et al have reported the presence of autoantibodies against SUMO-1 and SUMO-2 autoantigens in patients with primary biliary cirrhosis (PBC) (171). PBC is associated with other autoimmune conditions and several case reports have described PBC with co-existent myositis (172, 173).

SAE is a heterodimer composed of the subunits SAE1 and SAE2. Desterro et al have shown that the two polypeptides SAE1 and SAE2 have apparent molecular weights of 40 and 90 kDa respectively, with predicted molecular masses of 38 kDa and 72 kDa (174). These results are consistent with the data found in this study, where SAE2 was found to migrate at a higher molecular weight than predicted on SDS-PAGE. SAE functions in an ATP-dependant manner and forms a thioester bond between the SAE2 subunit and SUMO, prior to the transfer of SUMO to the E2 conjugating enzyme (174, 175). IIF staining using the SAE subunits have shown that SAE is distributed throughout the nuclei but excluded from the nucleoli (176). These results are consistent with the IIF staining patterns seen with anti-SAE positive sera, where a coarse speckled nucleolar sparing staining pattern was observed. The additional fine cytoplasmic speckle seen in some anti-SAE-positive cases suggests a limited cytosolic pool of the SAE, as previously shown with the sumoylation of RANGAP 1 (177).

Sumoylation is also implicated in the transcriptional repression of histone deacetylase a component of the nucleosome remodelling and deacetylase complex that also includes the myositis specific autoantigen, Mi-2 (178). Therefore, it is possible that the production of autoantibodies to either SAE or Mi-2 in DM maybe the consequence of the same post-translational modification event.

There are other protein targets that are modified by sumoylation (168). Intriguingly these include a member of the GATA family of transcription factors, GATA-2, which is expressed in adult endothelial cells. GATA-2 plays a key role in the transcriptional regulation of endothelial specific genes including endothelin-1 (ET-1), nitric oxide synthetase and Von-Willebrand factor. Both SUMO-1 and 2 covalently modify GATA-2 regulating ET-1 expression by suppressing promoter gene activity (179). This observation is of interest because endothelial cell injury and subsequent microvasculopathy in both muscle and skin lesions is well described in DM (74, 75, 180).
In summary, one can hypothesize that disruption of sumoylation of specific target proteins either via SUMO and/or the enzyme subunits SAE 1/2 play a role in the pathogenesis of certain DM clinical phenotypes. The clinical homogeneity of anti-SAE-positive patients, suggests that this is an important finding.

4.4.3 Anti-p155/140 autoantibodies

Over the last two years, several groups have reported an autoantibody termed anti-p155/140 based on the molecular weights of the polypeptide targets (155 and 140 kDa) in adult DM. Targoff et al and Kaji et al have identified this autoantibody specificity in 21% and 13% of DM patients respectively (143, 157). In both studies anti-p155/140 positive cases had more severe cutaneous involvement including more frequent V-sign and Shawl-sign rash, and very interestingly an association with malignancy. Furthermore, anti-p155/140 autoantibodies appear to be a negative predictor for interstitial lung disease. We have confirmed this observation in our cohort of adult DM patients. Chinoy et al investigated this further in larger cohort of patients from the AOMIC registry and found that the risk of malignancy in anti-p155/140 positive cases was significantly higher than anti-p155/140 negative cases (odds ratio of 23.2, 95% confidence interval 6.1-84.5) (158). These studies, and the work described in this chapter, highlight the future potential of routinely testing for anti-p155/140 to aid clinicians in the detection of cancer-associated myositis. The next step is to further validate the clinical utility of anti-p155/140, which can then be translated into routine clinical practice.

In this RNHRD cohort study only four patients out the overall IIM cohort had a history of malignancy and all were anti-p155/140 autoantibody positive. The diagnosis of cancer was either concurrent or within a few months onset of their DM, in concordance with the findings described by Kaji et al (157). Kaji et al also described two patients who were anti-p155/140-positive without malignancy and two patients who were clinically amyopathic although it is not clear if they were the same patients. Of interest, in this study, the three patients who were anti-p155/140 positive / malignancy negative also had CADM.

Preliminary work has identified the target of the anti-p155 autoantibodies as transcriptional intermediary factor 1-gamma (TIF1-γ) (159). Also known as Ret fused gene 7 (RFG7), this is a member of unique RING finger proteins involved in cellular differentiation (181). The discovery of the p155/140 autoantigen (TIF1-γ) in DM
patients with cancer-associated myositis may increase our understanding of the relationship between autoimmunity and cancer.
4.5 Appendix

I. Acknowledgements (including work done in conjunction with others)

Mrs J Dunphy (JO) and Mrs P Owen (PO) previously performed ANA IIF on sera from IIM cases as part of the RNHRD (Bath Institute for Rheumatic Diseases) Diagnostic Laboratory Service. I would like to thank them for giving me permission to include this data in this study.

I would also like to acknowledge and thank Dr Zoe Betteridge (ZB). The protein isolation, mass spectrometry work and immunodepletion experiments (anti-Zo and anti-SAE studies) were conducted by Dr Zoe Betteridge. I thank her for giving me permission to include her work in this thesis in conjunction with the clinical studies on anti-Zo and anti-SAE autoantibodies.

I would like to thank all the patients who have attended the RNRHD CTD clinic and given their consent to participate in these studies.
CHAPTER FIVE
RESULTS
Serological subsets and clinical associations of myositis-specific and myositis-associated autoantibodies in juvenile dermatomyositis

5.1 Introduction
5.1.1 Epidemiology
The juvenile idiopathic inflammatory myopathies (IIMs) are a group of rare but chronic systemic autoimmune conditions of childhood. Approximately one-fifth of all cases of IIM start in childhood, with an annual incidence of 2.5-5 cases per million population (19). Juvenile dermatomyositis (JDM) is the most common of the idiopathic inflammatory myopathies (IIM) of children, whereas juvenile polymyositis and inclusion body myositis are rarely described. The reported incidence of JDM ranges between 0.8-4.1 per million children per year (18-20). The peak age of onset is 7 years and most studies show that it is just over twice as common in girls than boys, although this ratio may be more comparable in patients under the age of 6 (19, 24). A younger age of onset may confer a worse overall prognosis and 25% cases occur before the age of 4 years (182).

5.1.2 Clinical features
JDM is chronic and potentially debilitating. Despite improvements in multi-disciplinary treatment approaches JDM is still associated with significant morbidity and mortality. Classic JDM rash is similar to adult DM lesions. Heliotrope rash, Gottron’s papules over the fingers or extensor aspects of the limbs, and periungual cuticular overgrowth with capillary dilatation are prominent features. Cutaneous lesions are the presenting feature in the majority (over 50% cases), and proximal muscle weakness is the first symptoms in a quarter (183). JDM is a multi-system disease and most children will have other ‘systemic’ manifestations at onset, including fever, weight loss, anorexia, mouth ulcers, alopecia and irritability (24). In comparison to adult DM, vasculitis is a more prominent feature leading to various clinical signs including subcutaneous oedema and subsequent skin ulceration. Other systemic involvement includes gastrointestinal (GI) vasculitis causing abdominal pain, GI ulceration, bleeding and perforation (24, 183). Dystrophic calcinosis (in the skin, subcutaneous tissue or muscle), acquired lipodystrophy and contractures are also relatively common manifestations in JDM, and are said to be associated with chronic cutaneous inflammation, longer disease duration and delay in diagnosis / treatment onset (184, 185). JDM-scleroderma overlap (JDM-SSc) is well recognised
in JDM children with a history of Raynaud’s phenomenon, sclerodactyly and other sclerodermatous skin changes. JDM can also overlap with systemic lupus erythematosus (JDM-SLE). In addition, it is well described that a large number of JDM children can also develop overlap inflammatory arthritis (usually in association with JDM-SSc or SLE) (24, 186). In contrast to adult DM, interstitial pneumonia and cancer-associated myositis are very rare in JDM (53, 55-57). Due to the heterogeneity of the condition with multi-organ involvement, clinical outcomes and prognosis are difficult to predict. Certain clinical features, such as skin ulceration, gastro-intestinal involvement (dysphagia and ischaemic ulceration from chronic endarteropathy) or respiratory disease / respiratory muscle weakness (especially leading to aspiration pneumonia) have been proposed as predictors of severe disease course in JDM (21, 25, 57, 187).

5.1.2 Clinical Assessment

There are now standardised validated clinical tools used in the assessment of JDM both in clinical practice and in clinical trials. These include the Childhood Myositis Assessment Scale (CMAS) and the Childhood Health Assessment Questionnaire (CHAQ) (188-191). Additional measures of disease activity include the Physicians’ global assessment using a visual analogue scale (VAS) or Likert scale, and a patient / parent global assessment of overall well-being using the same method (VAS) (192). In addition, the Cutaneous Assessment tool (CAT) has been partially validated for the assessment of skin disease in JDM (193, 194).

Standard laboratory markers include muscle enzymes; creatinine kinase (CK), lactate dehydrogenase (LDH), aldolase, and other inflammatory markers including C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR). Magnetic resonance imaging (MRI) of proximal muscle groups is now being frequently used for diagnostic purposes as well assessment of muscle disease activity, and in some cases may be preferred to muscle biopsy. T2 fat suppressed images can demonstrate muscle, subcutaneous and cutaneous inflammation and oedema. T1 weighted images are used to establish the degree of fatty infiltration and muscle atrophy, which is representative of damaged muscle (195-197).


**Childhood Myositis Assessment Scale (CMAS)** (188, 189)
Assessment comprises of a series of timed tasks testing muscle strength, endurance and function. See Chapter 5.5: Appendix I.

**Childhood Health Assessment Questionnaire (CHAQ)** (190, 191)
The CHAQ is a functional questionnaire completed by the patient and/or parent to assess activities of daily living. See Chapter 5.5: Appendix I.

**Cutaneous Assessment tool (CAT)** (193, 194)
The CAT is used in both adult and juvenile DM to assess skin disease measuring both activity and damage, using a priori weighting scale. See Chapter 5.5: Appendix I.

### 5.1.3 Aetiology and pathogenesis
The aetiology of JDM remains unclear, however similar to adult IIM, it is well recognised that genetic susceptibility combined with environmental triggers leads to the development of autoimmunity and thus immune dysfunction. The strongest genetic association for autoimmune connective tissue diseases, including JDM is the human leucocyte region (HLA) within the major histocompatibility complex (MHC). The MHC region consists of clustered multiple alleles coding for proteins central to the immune system. The 8.1 ancestral MHC haplotype, (HLA-B*08-DRB1*03-DQA1*05-DQB1*02) is associated with JDM, where the strongest signal appears to come from the HLA class I region (10, 198-200). Recent work demonstrates that the DQA1*0301 allele confers an additional risk for JDM, whereas certain alleles, DQA1*0201, DQA1*0101 and DQA1*0102 appear protective (200). Certain cytokine polymorphisms, including TNFα-0308A and IL-1 receptor antagonist, are also associated with Caucasian JDM with a higher risk of cutaneous ulceration and calcinosis (201, 202).

Environmental factors appear to play a role in the development of JDM. Reports suggest a temporal association to the disease onset. Various infectious agents have been implicated with the onset of JDM, in particular Group A streptococcus and several viruses including coxsackie, enterovirus and parvovirus (203-206). Seasonal birth patterns have been assessed in juvenile-onset IIM, in comparison to patients with adult-onset DM and healthy controls. It was noted that juvenile-onset IIM Hispanic patients had a seasonal birth pattern significantly different to controls. Birth patterns also differed in patients with certain HLA risk factors including...
DRB1*0301 and DQA1*0501 alleles. The authors concluded that birth distributions appear to have a stronger seasonality in juvenile IIM, suggesting a role for perinatal or early-life exposures (95).

Similar to adult IIM, both cellular and humoral mechanisms are involved in pathogenesis of JDM, and recent evidence suggests a role of the innate immune system (17, 73, 74, 207-210). Antibody-mediated complement deposition in the vessels plus dendritic cell (DCs), CD4+ T cell and B cell perivascular and perifascicular infiltration and upregulation of MHC class I and class II molecules by myocytes are characteristic. Vasculopathy plays a major pathophysiological role in JDM. Affected muscle is characterised by endothelial dysfunction and capillary dropout. Clinical features in the skin and GI tract suggest similar pathology (73). Hallmark features on muscle biopsy include oedema of the capillary endothelial wall with subsequent occlusive vasculopathy, perivascular and perimysial inflammatory cell infiltrate, muscle fibre changes including perifascicular atrophy. Evidence of muscle degeneration and regeneration is present with neonatal myosin. Recently an international consensus scoring system for the analysis of JDM muscle biopsy specimens has been published (211). Recent work has highlighted the importance of type 1 interferons (IFN) in JDM disease mechanisms. Type I IFN is believed to be critical in the propagation of autoimmunity, and this mechanism has been implicated in the pathogenesis of JDM as well as adult DM (212, 213). Studies using microarray technology have demonstrated up-regulation of type-1 IFN induced genes in DM (214, 215). DM muscle contains high numbers of gene transcripts associated with type 1 IFN, the source of which are CD4+ plasmacytoid dendritic cells (pDCs) that have chemokine activity. Similar findings were also noted in DM skin. Finally, the potential role of maternal microchimerism in JDM has been highlighted over the last few years. The presence of maternal cells in peripheral blood and diseased muscle of JDM cases may induce autoimmunity i.e. 'a graft-versus-host disease’ mechanism (216-218).

5.1.4 Autoantibodies in JDM and JDM-overlap syndromes
Until recently, unlike adult IIM, specific autoantibodies in juvenile IIM especially JDM, were detected infrequently despite the majority being ANA positive on IIF. As in adult IIM, autoantibodies are divided into MSAs and MAAs, the latter typically described in JDM-overlap syndromes (JDM-SSc and JDM-SLE).
Unlike adults the frequency of anti-aminoacyl tRNA synthetase (ARS) autoantibodies in juvenile myositis, in particular JDM, is much lower. Rider et al used immunodiffusion and immunoprecipitation (IPP) to screen sera from 77 children with myositis and overlap CTD, and anti-ARS were detected in only 2.6% cases (125). Using the same techniques, Feldman et al investigated 42 children; thirty five with JDM and 7 with other forms of IIM, and none had anti-ARS autoantibodies (127). The clinical features of anti-ARS-positive JDM cases are similar to their adult counterparts: moderate to severe weakness, arthritis, Raynaud's phenomenon, mechanic's hands, fevers and interstitial lung disease.

Similar to the ARS molecules, SRP is rarely targeted by autoantibodies in juvenile myositis. Based on previous studies the frequency of anti-SRP is 1% (125, 127) Recently, Rouster-Stevens et al described three cases of juvenile anti-SRP myositis in their cohort of 123 children and based on their clinical features the patients were comparable to adults with the same autoantibody specificity. In particular; all three children had severe weakness, very high creatinine kinase (CK) levels, were refractory to treatment and had myofibre necrosis on biopsy (219). Of interest, the ethnic origin of these patients was African American and Love at al has previously reported a higher frequency of anti-SRP in adult African American cases (6).

The reported frequency of anti-Mi-2 autoantibodies in JDM or JDM overlap is up to 5% (125, 127). Like adult DM, this autoantibody specificity is described in children with hallmark cutaneous DM lesions including Gottron’s papules, heliotrope rash, cuticular overgrowth and rashes on the neck and trunk. Moreover, patients may have milder muscle involvement, appear to have a monocyclic clinical course and respond well to therapy (125-127).

MAAs: anti-U1-RNP, anti-PM-Scl, anti-U3-RNP, anti-Ku and anti-topoisomerase are typically detected in children with JDM associated with systemic sclerosis features including scleroderma skin changes and Raynaud’s. Based on previous data, collectively the overall frequency in JDM / JDM-overlap is between 15-20% (57, 220, 221).

Previously, the overall frequency of defined MSAs and MAAs in children with IIM was between 20-40%, however using full serological testing, specifically IPP this frequency is now much higher following the studies described in the next two
chapters of this thesis, and earlier reports from Wedderburn et al, Targoff et al and Oddis et al (10, 143, 222).

The work described in this chapter is a clinical and serological study of JDM and JDM-overlap children recruited to the JDRR (UK and Ireland). Chapter 6 describes the work on novel autoantibody specificities in JDM.

5.2 Patients and Methods
5.2.1 Patients and sera
Subjects for the study described in this chapter were recruited from:
- The Juvenile Dermatomyositis Registry and Repository (JDRR), UK and Ireland (24).

The JDRR has recruited patients from 10 centres around the UK (for details see Chapter 5, Appendix III) with juvenile-onset myositis, below the age of 16 years at disease onset and diagnosis. All JDM cases had probable or definite disease according to Bohan and Peter criteria (1, 2). JDM-scleroderma overlap (JDM-SSc) was defined as JDM children with a history of Raynaud’s phenomenon, sclerodactyly and other sclerodermatous skin changes (two or more of the above features). Using a standardised proforma demographic and clinical data were recorded at diagnosis and prospectively at subsequent visits, on average every 6 months (see Chapter 2, Section 2.1.2 and 2.1.3, and appendix). The clinical information recorded included specific cutaneous manifestations including the presence of Gottron’s lesions, skin ulceration, oedema, calcinosis and the distribution of skin rash over the body. Details on muscle involvement included muscle enzymes (CK and LDH) and CMAS at disease baseline. The development of malignancy (within three years of diagnosis) was not specifically recorded on the clinical proforma. However, clinicians were able to enter free text data on clinically significant events during disease course, which was therefore likely to capture this information. Data were stored using anonymous codes onto a central database. Serum samples were taken at the time of diagnosis and stored at -20°C until required.

5.2.2 Serological Methods
ANA-Immunofluorescence (IIF)
IIF was previously performed by standard methods using HEp-2 cells and fluorescein-labelled anti-human IgG immunoglobulin (Sigma, UK).
**Protein Radio-immunoprecipitation (IPP)**

Sera stored at -20°C were thawed at room temperature. IPP from K562 cell extracts was performed as described in Chapter 2, see Sections 2.3.2 and 2.3.3. Briefly, 10 µl sera was mixed with 2 mg protein-A-Sepharose beads (Sigma, UK) in IPP buffer (10 mM Tris-Cl pH 8.0, 500 mM NaCl, 0.1% v/v Igepal) at room temperature for 30 min. Beads were washed in IPP buffer prior to the addition of 120 µl [³⁵S] methionine labelled K562 cell extract. Samples were mixed at 4°C for 2 hr. Beads were washed in IPP buffer followed by TBS buffer (10 mM Tris-Cl pH 7.4, 150 mM NaCl) before being resuspended in 50 µl SDS sample buffer (Sigma, UK). After heating, proteins were fractionated by 10% SDS-PAGE, enhanced, fixed and dried. Labelled proteins were analysed by autoradiography.

5.2.3 Ethical approval

All patients gave fully informed written / parental consent to participate and provide biological samples according to the Declaration of Helsinki under both national multi-centre and local ethical committee regulations.

5.2.4 Statistical Analysis

Clinical associations were derived from 2×2 contingency tables using the chi-squared test, or two-tailed Fisher’s exact test where individual cells valued five or less. P values less than 0.05 were considered as significant (uncorrected P values presented in view of small numbers in each autoantibody group). SPSS for Windows (version 14) was used to perform statistical analysis.

5.3 Results

Serum samples for serological typing were available from 162 children recruited to the registry. Full clinical data were available on 160 children and 74% were female. The median age of disease onset was 6 years, inter-quartile range (IQR) 3-9 and median age at diagnosis was 7 years (IQR 4, 10). One hundred and twenty seven cases were Caucasian and 35 cases were non-Caucasian.

The median follow-up from disease onset to the time of data analysis for this study was 48 months (IQR 33, 72) for the overall cohort.

One hundred and thirty seven children had JDM. In this study, 21 children were defined as JDM-SSc. Two children were defined as having indeterminate juvenile myositis, not specifically JDM or JDM-SSc.
5.3.1 Serological profiles
Following ANA IIF and IPP, autoantibody specificity was categorised into recognised MSAs, myositis-associated autoantibodies (MAAs), unidentified / novel or negative. The overall frequency of previously defined MSAs and MAAs in this cohort study is shown in Table 14. Table 15 shows a breakdown of serological results and ethnicity (Caucasian and non-Caucasian).

IPP results
Thirteen out of 162 (8%) cases were positive for previously described MSAs: anti-Jo-1 (1.2%) and anti-Mi-2 (6.8%). No cases were positive for anti-SRP autoantibodies. Anti-Mi-2 was found exclusively in JDM patients, with a frequency of 7.9%, and not in any JDM-overlap patients. Anti-Jo-1 was found in JDM-overlap only. The frequency of ‘known’ MSA-positive in JDM was 8% compared to 9.5% in JDM-overlap.

The overall frequency of MAAs in this study cohort was 14.2%: anti-U1-RNP (5.6%), anti-PM-Scl (6.8%), anti-U3-RNP (0.6%), anti-Ku (0.6%) and anti-Topoisomerase (0.6%). There was a much higher frequency of MAA-positive cases in JDM-overlap (81%) compared to MAA-positive JDM (4.4%). Of note, anti-U1-RNP and anti-PM-Scl was found in higher frequency in Caucasians compared to non-Caucasian children.

Novel autoantibodies were detected in 40% of the overall cohort, and these specificities were found exclusively in JDM cases with a frequency of 47.4% (this work is described in detail in Chapter 6). In the overall cohort, 37.6% of cases were either negative or had undefined bands on IPP.

See Figures 15A-C for examples of MAAs and MSAs in JDM study.

For full IPP results see Chapter 5.5, Appendix II.

ANA IIF results
For overall results see Chapter 5.5, Appendix II.

In total, ANA IIF was performed on 124 samples (there were insufficient sera in 38 samples for both ANA IIF and IPP, therefore only IPP was performed). Out of 124 cases, 96 (77.4%) were ANA positive by IIF.
Figure 15A: Immunoprecipitation of MSAs and MAAs in JDM cohort study
10% SDS PAGE of immunoprecipitates of [35S] labelled K562 cell extracts.
JDM sera used for immunoprecipitation: Lane 1; anti-Mi-2, Lane 5; anti-Mi-2, Lane 7; anti-Mi-2, Lane 9; anti-U1-RNP.
Figure 15B: Immunoprecipitation of MSAs and MAAs in JDM cohort study
10% SDS PAGE of immunoprecipitates of [35S] labelled K562 cell extracts.
JDM sera used for immunoprecipitation: Lane 1; anti-U1-RNP, Lanes 2 and 9; anti-PM-Scl, Lane 3 and 5; novel anti-p155/140 (see Chapter 6), Lane 4; possible anti-U1-RNP, Lane 6; novel anti-p140 (see Chapter 6).
Figure 15C: Immunoprecipitation of MSAs and MAAs in JDM cohort study
10% SDS PAGE of immunoprecipitates of \( ^{35}S \) labelled K562 cell extracts.
JDM sera used for immunoprecipitation: Lane 1; anti-Ku and anti-U3-RNP, Lane 3;
anti-Mi-2, Lane 7; novel anti-p140 (see Chapter 6), Lane 11; anti-U1-RNP.
### Table 14: Myositis-specific autoantibodies and Myositis-associated autoantibodies in JDM and JDM-overlap cohort

<table>
<thead>
<tr>
<th>Autoantibody</th>
<th>JDM n=137</th>
<th>JDM-overlap n=21</th>
<th>Other myositis n=2</th>
<th>Overall n=162</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MSAs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-ARS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Jo-1</td>
<td>0</td>
<td>9.5</td>
<td>0</td>
<td>1.2*</td>
</tr>
<tr>
<td>Anti-PL-12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-PL-7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-EJ</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-OJ</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-KS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-Ha</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-Zo</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-SRP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-Mi-2</td>
<td>7.9</td>
<td>0</td>
<td>0</td>
<td>6.8</td>
</tr>
<tr>
<td><strong>MAAs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-U1-RNP</td>
<td>2.2</td>
<td>28.6</td>
<td>0</td>
<td>5.6</td>
</tr>
<tr>
<td>Anti-PM-Scl</td>
<td>2.2</td>
<td>38.1</td>
<td>0</td>
<td>6.8</td>
</tr>
<tr>
<td>Anti-U3-RNP</td>
<td>0</td>
<td>4.8</td>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td>Anti-Ku</td>
<td>0</td>
<td>4.8</td>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td>Anti-Topo</td>
<td>0</td>
<td>4.8</td>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>Novel antibodies</strong></td>
<td>47.4</td>
<td>0</td>
<td>0</td>
<td>40.1</td>
</tr>
<tr>
<td>See Chapter 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Unknown bands</strong></td>
<td>16.1</td>
<td>9.5</td>
<td>50</td>
<td>15.4</td>
</tr>
<tr>
<td><strong>Negative</strong></td>
<td>25.5</td>
<td>0</td>
<td>50</td>
<td>22.2</td>
</tr>
</tbody>
</table>

Values shown are in percentages

MSAs: myositis-specific autoantibodies
MAAs: myositis-associated autoantibodies
Topo: Topoisomerase (Scl-70)
*One anti-Jo-1-positive case also positive for anti-Ro autoantibodies
Table 15: Myositis-specific autoantibodies and Myositis-associated autoantibodies in JDM and JDM-overlap cohort (ethnic groups)

<table>
<thead>
<tr>
<th>Autoantibody</th>
<th>JDM Caucasian n=108</th>
<th>JDM Non-Caucasian n=31</th>
<th>JDM-overlap Caucasian n=19</th>
<th>JDM-overlap Non-Caucasian n=4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MSAs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-ARS (Jo-1)</td>
<td>0</td>
<td>0</td>
<td>10.5</td>
<td>0</td>
</tr>
<tr>
<td>Anti-SRP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-Mi-2</td>
<td>8</td>
<td>6.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>MAAs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-U1-RNP</td>
<td>1.9</td>
<td>0</td>
<td>31.6</td>
<td>25</td>
</tr>
<tr>
<td>Anti-PM-Scl</td>
<td>0.8</td>
<td>6.5</td>
<td>42.1</td>
<td>0</td>
</tr>
<tr>
<td>Anti-U3-RNP</td>
<td>0</td>
<td>0</td>
<td>5.3</td>
<td>0</td>
</tr>
<tr>
<td>Anti-Ku</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Anti-Topo</td>
<td>0</td>
<td>0</td>
<td>5.3</td>
<td>0</td>
</tr>
<tr>
<td><strong>Novel antibodies</strong></td>
<td>42.6</td>
<td>61.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Unknown bands</strong></td>
<td>17.6</td>
<td>9.7</td>
<td>5.3</td>
<td>25</td>
</tr>
<tr>
<td><strong>Negative</strong></td>
<td>27.8</td>
<td>16.1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values shown are in percentages
MSAs: myositis-specific autoantibodies / MAAs: myositis-associated autoantibodies
Topo: Topoisomerase (Scl-70)
5.3.2 Clinical associations of myositis autoantibodies in study cohort

The overall clinical features of the entire cohort are shown in Table 16. The main clinical features of MSAs (anti-Jo-1 and –Mi-2) and MAAs are shown in Table 17. Two cases were anti-Jo-1-positive with JDM-overlap. The median age at onset was 9 years and both cases were females. Both children had DM rash with myositis, arthritis, sclerodermatous puffy fingers and Raynaud’s phenomenon. This MSA subset had no complicating features such as skin ulceration or calcinosis, however the numbers are small. One anti-Jo-1-positive child also had interstitial pneumonia and mechanic’s hands.

Anti-Mi-2 was detected in 11 children and all had JDM with hallmark cutaneous changes and a wide distribution of rash: Gottron’s papules (100%), cutaneous oedema 63%, periorbital (100%) and periungual overgrowth with capillary changes (91%). The median age at onset was 12.5 years and 90% cases were girls.

MAAs (specifically anti-U1-RNP, -PM-Scl, -U3-RNP, -Ku, -Topo) were detected in 17 children with JDM-overlap and 6 children with a diagnosis of JDM. MAA-positive children had a median age at onset of 9 years, and 78% were female. Sixty-five% of MAA-positive cases had Gottron’s papules, with periorbital rash (35%), periungual changes (57%) and rash on the trunk (17%). Other features were frequent: arthritis (65%), scleroderma skin changes (61%), Raynaud’s (48%), calcinosis (21%) and lipoatrophy (35%). Both anti-PM-Scl and anti-U1-RNP autoantibodies were present in 5-7% of juvenile IIM respectively (a similar frequency to anti-Mi-2).

When comparing anti-Mi-2-positive children with MAA-positive children the frequency of skin lesions - Gottron’s papules (P=0.034) and cutaneous oedema (P=0.021), distribution of rash – periorbital (P<0.001), were significantly higher in the anti-Mi-2 group. Sclerodermatous features were significantly more frequent in the MAA-positive group (P<0.001).
Table 16: Overall clinical features of the UK JDM Cohort study (n=160)

<table>
<thead>
<tr>
<th>Clinical feature</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>73.8</td>
</tr>
<tr>
<td>Caucasian</td>
<td>79.4</td>
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</table>

**Skin disease**
- Gottron’s: 82.9
- Ulceration: 25.3
- Lipoatrophy: 14
- Oedema: 34.8
- Calcinosis: 23.3

**Distribution of rash**
- Periorbital: 71.4
- Periungal: 66.2
- Trunk: 14.3
- Small joints: 70.8
- Large joints: 51.3

**Other features**
- Arthritis: 44.7
- Sclerodermatous features: 12.6
- Raynaud’s phenomenon: 16.6
- Dysphagia: 31.4
- Mouth ulcers: 27
- Alopecia: 29.7
- Interstitial pneumonia: 5

Values are in percentages
Table 17: Clinical features of MSAs and MAAs in JDM and JDM-overlap cohort

<table>
<thead>
<tr>
<th></th>
<th>Anti-Jo-1 n=2 (%)</th>
<th>Anti-Mi-2 n=11 (%)</th>
<th>MAA n=23 (%)</th>
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<td>12.5</td>
<td>7</td>
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<tr>
<td>Median age</td>
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<td>9</td>
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<td>(diagnosis)</td>
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<tr>
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<td>65 *</td>
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<td>13</td>
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<td>Oedema</td>
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<td>17 **</td>
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<td>Mechanic’s hands</td>
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<td>0</td>
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</tr>
<tr>
<td>Periorbital</td>
<td>100</td>
<td>100 δ</td>
<td>35 δ</td>
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<tr>
<td>Periungal</td>
<td>100</td>
<td>91</td>
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<tr>
<td>Small joints</td>
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<td>Trunk</td>
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<tr>
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<td>36</td>
<td>65</td>
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<tr>
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<td>100</td>
<td>0 α</td>
<td>61α</td>
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<td>9</td>
<td>48</td>
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<tr>
<td>Dysphagia</td>
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<td>45</td>
<td>30</td>
</tr>
<tr>
<td>Mouth ulcers</td>
<td>0</td>
<td>36 αα</td>
<td>4 αα</td>
</tr>
<tr>
<td>Alopecia</td>
<td>100</td>
<td>30</td>
<td>26</td>
</tr>
<tr>
<td>Interstitial pneumonia</td>
<td>50</td>
<td>0</td>
<td>4</td>
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<tr>
<td>Myositis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raised CK</td>
<td>100</td>
<td>70</td>
<td>76</td>
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</tbody>
</table>

Values are in percentages, unless otherwise stated, (n/a: full data not available)

* $P_{(uncorr)}=0.034$: anti-Mi-2-positive versus MAA-positive

** $P_{(uncorr)}=0.021$: anti-Mi-2-positive versus MAA-positive

δ $P<0.001$: anti-Mi-2-positive versus MAA-positive

α $P<0.001$: anti-Mi-2-positive versus MAA-positive

αα $P_{(uncorr)}=0.05$: anti-Mi-2-positive versus MAA-positive
5.4 Discussion

This chapter describes a serological study of JDM and JDM-overlap in children recruited to the JDRR. The aim of the work in this chapter was to establish the frequency of known MSAs and MAAs, and describe the clinical associations in this JDM UK cohort.

In comparison to previous serological studies in juvenile IIM summarised in several review articles (221, 223, 224), the overall frequency of MSAs and MAAs are similar, especially the anti-Mi-2 and MAAs subsets. In a recent study by Espada et al, 64 Argentine Caucasian children with juvenile IIM (including 40 JDM cases and 17 myositis overlap cases) were screened using IPP and RNA immunoprecipitation. The frequency of anti-Mi-2 was 6%, anti-PM-Scl was 3% and anti-U1-RNP autoantibody was 11%. No children were positive for anti-ARS autoantibodies (225). In this cohort study, the frequency of anti-ARS autoantibodies was generally less than previously described (125, 127), with less anti-Jo-1-positives and no children testing positive for non-Jo-1-ARS. In contrast to adult IIM where autoantibodies to ARS are a common target (as described in Chapter 3), this group of MSAs are detected infrequently in juvenile myositis. Although the numbers of children with anti-ARS autoantibodies were small, the clinical manifestations do resemble that of their adult counterparts with the anti-synthetase syndrome (see Chapter 3) (46). Anti-SRP autoantibodies were not detected in this cohort, which may reflect the ethnic make-up of this cohort (the majority of cases being Caucasian) with no cases of ‘juvenile PM’ being recruited to the JDRR at the time of this study. Typically anti-SRP-positivity is rare in juvenile IIM anyway and a recent report has highlighted how this MSA may be generally more prevalent in Afro-American children (219). Anti-Mi-2 was detected in JDM children with hallmark cutaneous changes, a wide distribution of rash, and a relatively higher frequency of cutaneous / subcutaneous oedema in comparison to the anti-Jo-1 and MAA subgroups. In contrast, extra-muscular / cutaneous complications were relatively less frequent except for dysphagia. The frequency of anti-Mi-2 in both juvenile and adult myositis is similar.

The frequency of MAAs in this juvenile cohort is similar to that seen in adult IIM, especially when comparing JDM-SSc patients with adult myositis/CTD overlap (139). The most common MAA specificities were anti-U1-RNP and anti-PM-Scl, with small number of children testing positive for anti-U3-RNP, -Topo, and -Ku. There were no patients with other systemic sclerosis-associated autoantibodies e.g. anti-centromere or anti-RNA polymerase I, II or III, which highlights differences between
juvenile and adult myositis-SSc overlap populations (139, 226). Children with SSc, including the diffuse form of the disease, have similar serological profiles. Anti-PM-Scl and anti-U1-RNP autoantibodies are detected in a higher frequency compared to adult onset SSc. Other specificities including anti-centromere, anti-RNA polymerase and anti-topoisomerase are more common in adult SSc (226, 227). In general, MAA-positive children had less classic skin DM lesions but myositis with more arthritis, scleroderma skin changes, Raynaud’s and lipoatrophy. In comparison to anti-Jo-1 and anti-Mi-2-positive children, MAA-positives had a relatively higher frequency of calcinosis, and this observation may reflect the predominant overlap features with scleroderma. In general, overlap features are seen in about approximately 10% of juvenile-onset IIM (21, 22). In this study, 12% of children were defined as having JDM-SSc overlap, and although full data was not available to make any conclusions regarding severity of muscle disease, it has been observed that children with overlap may have milder muscle disease with lower disease activity markers (21).

In summary, the overall frequency of recognised MSA or MAAs in this cohort was around 20%. Therefore, most children with myositis are either autoantibody negative or have autoantibodies that target novel autoantigens. The majority have novel specificities and this work is described in Chapter 6.
## ChildHOod Myositis Assessment Scale (CMAS) Scoring Sheet

1. **Head Lift:**
   - 0 = Unable
   - 1 = 1-9 sec
   - 2 = 10-29 sec
   - 3 = 30-59 sec
   - 4 = 60-119 sec
   - 5 = ≥ 2 min

2. **Leg Raise/Touch Object:**
   - 0 = Unable to lift leg off table.
   - 1 = Able to clear table, but cannot touch object (examiner’s hand).
   - 2 = Able to lift leg high enough to touch object (examiner’s hand).

3. **Straight Leg Lift Duration:**
   - 0 = Unable
   - 1 = 1-9 sec
   - 2 = 10-29 sec
   - 3 = ≥ 2 min

4. **Squat to Stand:**
   - 0 = Unable
   - 1 = Easily returns to squat position
   - 2 = Some difficulty
   - 3 = Severe difficulty

5. **Sit-Ups:**
   - 0 = Unable
   - 1 = With counterbalance
   - 2 = Without counterbalance

6. **Squat to Sit:**
   - 0 = Unable
   - 1 = Almost unable
   - 2 = Slight difficulty
   - 3 = No difficulty

7. **Arm Raise/Height:**
   - 0 = Cannot raise arms to the level of the A-C point.
   - 1 = Can raise arms at least to the level of the A-C point, but not above top of head.
   - 2 = Can raise arms above top of head, but cannot raise arms straight above head or that allowers are in full extension.
   - 3 = Can raise arms straight above head so that allowers are in full extension.

8. **Arm Raise Duration:**
   - 0 = Unable
   - 1 = 1-9 sec
   - 2 = 10-29 sec
   - 3 = ≥ 2 min

The maximum possible total score for the 14 maneuvers is 82 (32 points of muscle strength function).

**Patient:____________ Date:__________**

**Total CMAS Score:______**
### CHILDHOOD HEALTH ASSESSMENT QUESTIONNAIRE

In this section we are interested in learning how your child's illness affects his/her ability to function in daily life. Please feel free to add any comments on the back of this page. In the following questions, please check the one response which best describes your child’s usual activities (averaged over an entire day) **OVER THE PAST WEEK**, ONLY NOTE THOSE DIFFICULTIES OR LIMITATIONS WHICH ARE DUE TO ILLNESS. If most children at your child's age are not expected to do a certain activity, please mark it as "Not Applicable". For example, if your child has difficulty in doing a certain activity or is unable to do it because he/she is too young but not because he/she is RESTRICTED BY ILLNESS, please mark it as "NOT Applicable".

<table>
<thead>
<tr>
<th>Without ANY Difficulty</th>
<th>With SOME Difficulty</th>
<th>With MUCH Difficulty</th>
<th>UNABLE To do</th>
<th>Not Applicable</th>
</tr>
</thead>
</table>

#### DRESSING & GROOMING

5. Is your child able to:
   - Dress, including tying shoeaces and doing buttons?
   - Shampoo his/her hair?
   - Remove socks?
   - Cut fingernails?

#### ARISING

10. Is your child able to:
    - Stand up from a low chair or floor?
    - Get in and out of bed or stand up in a crib?

#### EATING

15. Is your child able to:
    - Cut his/her own meat?
    - Lift up a cup or glass to mouth?
    - Open a new cereal box?

#### WALKING

20. Is your child able to:
    - Walk outdoors on flat ground?
    - Climb up five steps?

23. * Please check any AIDS or DEVICES that your child usually uses for any of the above activities:
    - Cane
    - Stool
    - Walker
    - Special or built up chair
    - Wheelchair
    - Built up pencil or special utensils
    - Other (Specify:______________)

28. * Please check any categories for which your child usually needs help from another person BECAUSE OF ILLNESS:
   - Dressing and Grooming
   - Eating
   - Walking
<table>
<thead>
<tr>
<th></th>
<th>Without ANY Difficulty</th>
<th>With SOME Difficulty</th>
<th>With MUCH Difficulty</th>
<th>UNABLE to do</th>
<th>Not Applicable</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**HYGIENE**

32. Is your child able to:
   33. Wash and dry entire body?  
   34. Take a tub bath (get in and out of tub)?  
   35. Get on and off the toilet or pony chair?  
   36. Brush teeth?  
   37. Comb/brush hair?  

**REACH**

38. Is your child able to:
   39. Reach and get down a heavy object such as a large game or books from just above his/her head?  
   40. Bend down to pick up clothing or a piece of paper from the floor?  
   41. Pull on a sweater over his/her head?  
   42. Turn neck to look back over shoulder?  

**GRIP**

43. Is your child able to:
   44. Write or scribble with pen or pencil?  
   45. Open car doors?  
   46. Open jars which have been previously opened?  
   47. Turn faucets on and off?  
   48. Push open a door when he/she has to turn a door knob?  

**ACTIVITIES**

49. Is your child able to:
   50. Run errands and shop?  
   51. Get in and out of a car or toy car or school bus?  
   52. Ride bike or tricycle?  
   53. Do household chores (e.g. wash dishes, take out trash, vacuum, yardwork, make bed, clean room)?  
   54. Run and play?  

55. Please check any AIDS or DEVICES that your child usually uses for any of the above activities:
   56. Raised toilet seat  
   57. Bathtub seat  
   58. Jar opener (for jars previously opened)  
   59. Long-handled appliances for reach  
   60. Long-handled appliances in bathroom

**PAIN:** We are also interested in learning whether or not your child has been affected by pain because of his/her illness.

61. How much pain do you think your child has had because of his/her illness IN THE PAST WEEK?
   Place a mark on the line below, to indicate the severity of the pain
   62. No pain 0 ———————————————————— 100 Very severe pain

**GLOBAL EVALUATION:** Considering all the ways that arthritis affects your child, rate how he/she is doing by placing a single mark on the line below.

63. Very well 0 ———————————————————— 100 Very poor

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5.5 Appendix I

Cutaneous Assessment Tool

Full proforma available on:

http://dir-apps.niehs.nih.gov/imacs/index.cfm?action1/4home.main
### 5.5 Appendix II – Subjects, ANA IIF and IPP results

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<th>ANA IIF result</th>
<th>IPP result</th>
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FSNS – fine speckle nucleolar sparing
DCNS – diffuse coarse nucleolar sparing
γ - ANA IIF not performed (insufficient serum available)
5.5 Appendix III

Acknowledgements

I would like to thank Mrs J Dunphy and Mrs P Owen for their assistance and guidance in performing ANA IIF on the juvenile myositis samples. Thank you to Dr Zoe Betteridge for her guidance with my immunoprecipitation studies. I would also like to thank Prof Lucy Wedderburn, Scientific Director for the Juvenile Dermatomyositis UK Cohort Study for her collaboration, guidance and support. On behalf of the Juvenile Dermatomyositis Research Group I would like to thank all the patients and their families who contributed to the Juvenile Dermatomyositis Cohort Study. I thank all local research coordinators and principal investigators who have made this research not have been possible. The members who contributed were: Mr Ian Roberts, The Royal Liverpool Children’s Hospital, Alder Hey, Liverpool, and Booth Hall Children’s Hospital, Manchester; Dr Eileen Baildam, Booth Hall Children’s Hospital, Manchester and now Alder Hey, Liverpool; Dr Phil Riley, Booth Hall Children’s Hospital, Manchester; Mrs Janis Scott and Dr Clive Ryder, Birmingham Children’s Hospital, Birmingham; Mrs Gillian Jackson and Dr Sue Wyatt, Leeds General Infirmary, Leeds; Ms Elizabeth Camp and Dr Janet Gardner-Medwin, The Royal Hospital for Sick Children, Yorkhill, Glasgow; Mrs Alison Swift, Dr Helen Foster and Dr Mark Friswell, The Royal Victoria Infirmary, Newcastle; Mrs Elizabeth Hutchinson and Dr Helen Venning, Queens Medical Centre, Nottingham and Dr Clarissa Pilkington, Dr N Hasson and Ms Sue Maillard, Great Ormond Street Hospital, London. I thank H Varsani for technical assistance, in particular preparation of the serum samples, which were sent to the RNHRD for this study. The Arthritis Research Campaign awarded me the Barbara Ansell fellowship in Paediatric Rheumatology (grant 18136) to perform this work. I am grateful to the Arthritis Research Campaign who funded the autoantibody testing for this study as part of my fellowship.
CHAPTER SIX
RESULTS
Identification and characterisation of novel autoantigen systems in juvenile dermatomyositis

6.1 Introduction
Classifying patients using a clinico-serological approach may lead to the identification of more homogeneous subsets within the JDM spectrum and therefore have prognostic implications.

The work described in Chapter 5 demonstrates that around 20% of children with JDM or JDM-overlap have well-defined autoantibody specificities (anti-ARS, anti-Mi-2 and MAAs). Therefore, approximately 80% of children would normally be serologically classified as either being non-specifically ANA positive or in some cases seronegative, when tested by ANA IIF or standard ENA ELISA. Over the last few years, a number of novel autoantibodies have been described in adult IIM (as outlined in Chapters 3 and 4). Similarly, there has been a preliminary report from Oddis et al of a new autoantibody termed anti-MJ, which targets a ~140-142 kDa protein in JDM (222). A further autoantigen target with a molecular weight of 155 kDa (reported in most cases as a doublet protein with a second weaker band at 140 kDa) has been described in JDM in a study by Targoff et al (143). The same novel specificity anti-p155/140 is detected in adult DM, which was described in the same study (143) and was also seen in the RNHRD adult IIM study (see Chapter 4). Two further studies have described anti-p155/140 in separate adult DM cohorts (157, 158). As previously discussed in Chapter 4, anti-p155/140 in adult DM is associated with more severe skin disease but more significantly has a strong association with malignancy. In the Targoff et al study, anti-p155/140 was seen in approximately 30% of their JDM population, however the clinical specificity of this cohort was not described (143).

The purpose of the work described in this chapter was to establish the frequency and to define the clinical significance of anti-p155/140 and anti-p140 autoantibodies in children recruited to the UK JDM Cohort Study. The secondary aims were to confirm whether the same p155/140 autoantigen is targeted in adult DM and to demonstrate p155/140 and p140 are different targets. As presented in Chapter 5, 40% of patients tested by IPP were found to be positive for these two different
autoantibodies. Both autoantibodies appear to define distinct clinical phenotypes within the JDM spectrum. This work has been published in the following journals:


6.2 Patients and Methods

6.2.1 Patients and sera

Subjects for the study described in this chapter were recruited from:

- The Juvenile Dermatomyositis Registry and Repository, UK and Ireland (24).

Full details are given in Chapter 5 (see Section 5.2.1). In brief, the JDRR has recruited patients from 10 centres around the UK (for details see appendix (24)) with juvenile-onset myositis, below the age of 16 years at disease onset and diagnosis, to form the JDM UK Cohort Study. All JDM cases had probable or definite disease according to Bohan and Peter criteria (1, 2). Demographic and clinical data using a standardised proforma were recorded at diagnosis and prospectively at subsequent visits, on average every 6 months (see Chapter 2, Section 2.1.2 and 2.1.3, and Appendix). Data were stored using anonymous codes onto a central database. Serum samples were taken at the time of diagnosis and stored at -20°C until required.

6.2.1.1 Study 1 (Anti-p155/140 study)

The first 116 patients recruited to the JDM National Registry and Repository (UK and Ireland) (now termed the JDM UK Cohort Study) were initially studied. The median age at symptom onset was 6 years (IQR 3, 9) and at diagnosis was 7 years (IQR 4, 10). The median follow-up from disease onset to time of this study was 69.5 months (IQR 47.5, 105.2) and 44.2 months (IQR 35.7, 65.8) from date of entry into
the registry to time of this study. Clinical information included the degree of skin involvement; muscle strength tested by the Childhood Myositis Assessment Scale (CMAS) (see Chapter 5, Appendix) (188), Physician’s 10-point global assessment (PGA) (visual analogue scale) and serum muscle enzymes. When evaluating muscle involvement, only those JDM patients with documented muscle enzymes at disease onset / diagnosis (with subsequent maximum CK or LDH during follow up) were included for analysis. Similarly, those with a baseline CMAS or PGA at the time of entry to the registry (plus subsequent serial measures) were included. The type of skin lesion, in particular skin ulceration, oedema and Gottron’s lesions combined with the distribution (extent) of skin involvement plus CMAS/PGA score was defined as a marker of disease severity for the purpose of this study.

6.2.1.2 Study 2 (Anti-p140 study)

Patients

Serum samples for serological typing were available from the overall cohort of 162 children recruited to the registry (including the first 116 children initially recruited / analysed in the anti-p155/140 study). Clinical data were available on 160 children and 74% were female. The median age of disease onset was 6 years, inter-quartile range (IQR) 3-9 and median age at diagnosis was 7 years (IQR 4, 10). The median follow-up from disease onset to the time of data analysis for this study was 48 months (IQR 33, 72) for the overall cohort. One hundred and thirty seven children had JDM. JDM-scleroderma overlap (JDM-SSc) is well recognised in JDM children with a history of Raynaud’s phenomenon, sclerodactyly and other sclerodermatous skin changes. In this study, 21 children were defined as JDM-SSc with two or more of the above features. Two children were defined as having other forms of juvenile myositis, not specifically JDM or JDM-SSc.

Controls

Sera from 124 juvenile disease controls; twenty scleroderma / linear scleroderma, 8 systemic lupus erythematosus and 96 juvenile idiopathic arthritis cases were also analysed. No sera from healthy children were available, reflecting ethical difficulties in studies of this nature. Therefore, sera from 50 healthy adult controls were also serotyped.
6.2.2 Serological methods

Study 1 and 2

Indirect immunofluorescence (IIF)

IIF was performed by standard methods using HEp-2 cells and fluorescein-labelled anti-human IgG immunoglobulin (Sigma, UK).

Protein Radio-Immunoprecipitation (IPP)

IPP from K562 cell extracts was performed as previously described in detail in Chapter 2. Briefly, 10 µl sera was mixed with 2 mg protein-A-Sepharose beads (Sigma, UK) in IPP buffer (10 mM Tris-Cl pH 8.0, 500 mM NaCl, 0.1% v/v Igepal) at room temperature for 30 min. Beads were washed in IPP buffer prior to the addition of 120 µl \[^{35}\text{S}\] methionine labelled K562 cell extract. Samples were mixed at 4°C for 2 hr. Beads were washed in IPP buffer followed by TBS buffer (10 mM Tris-Cl pH 7.4, 150 mM NaCl) before being resuspended in 50 µl SDS sample buffer (Sigma, UK). After heating, proteins were fractionated by 10% SDS PAGE, enhanced, fixed and dried. Labelled proteins were analysed by autoradiography.

Immunodepletion experiments

Study 1

Immunodepletion experiments were performed using reference anti-p155/140-JDM-positive sera and reference anti-p155/140-adult-positive sera (adult sera from the RHHRD serology study – see Chapter 4, Section 4.3.4), to ascertain if the IPP pattern seen was due to precipitation of the same p155/140 autoantigen. Cell extracts were depleted of autoantibody targets using anti-p155/140-JDM-positive serum or anti-p155/140-adult DM-positive serum and normal serum as a negative control. These extracts were then used in further immunoprecipitations using both juvenile and adult anti-p155/140 positive serum.

As described in Chapter 2, Section 2.3.4: duplicate samples each containing 10 mg protein A Sepharose beads in 1 ml IPP buffer and 50 µl reference anti-p155/140 serum were mixed with end-over-end rotation at room temperature for 30 min. Beads were washed four times in 1 ml IPP buffer and 1 tube (A) was placed on ice whilst 150 µl \[^{35}\text{S}\] methionine-labelled K562 cell extract and 350 µl IPP buffer was added to the remaining tube (B). Tube B was mixed at 4°C for 2 hr after which the supernatant was transferred to tube A, this was mixed at 4°C for a further 2 hr. The supernatant from tube A was then transferred to a fresh tube (C) and stored at -80°C. IPP using JDM or adult DM serum and either 150µl control \[^{35}\text{S}\]-methionine-
labelled cell extract or the immunodepleted supernatants (C) were completed as described for IPP using $[^{35}\text{S}]$ methionine.

**Study 2**

*Immunodepletion experiments with anti-p140-JDM and anti-p155/140-JDM sera*

Immunodepletion experiments were performed using reference anti-140-JDM-positive sera and reference anti-p155/140-JDM-positive sera to confirm that these autoantibodies target different autoantigens. Cell extracts were depleted of autoantibody targets using anti-140-JDM positive serum or anti-p155/140-JDM positive serum and normal serum as a negative control. These extracts were then used in further immunoprecipitations using different JDM anti-p140 and anti-p155/140 positive serum. As described in Chapter 2, Section 2.3.4: duplicate samples each containing 10 mg protein A Sepharose beads in 1 ml IPP buffer and 50 µl reference anti-p140 or reference anti-p155/140 serum were mixed with end-over-end rotation at room temperature for 30 min. Beads were washed four times in 1 ml IPP buffer and 1 tube (A) was placed on ice whilst 150 µl $[^{35}\text{S}]$ methionine-labelled K562 cell extract and 350 µl IPP buffer was added to the remaining tube (B). Tube B was mixed at 4°C for 2 hr after which the supernatant was transferred to tube A, this was mixed at 4°C for a further 2 hr. The supernatant from tube A was then transferred to a fresh tube (C) and stored at -80°C. IPP using JDM serum and either 150µl control $[^{35}\text{S}]$-methionine-labelled cell extract or the immunodepleted supernatants (C) were completed as described for IPP using $[^{35}\text{S}]$ methionine.

*IPP with mouse monoclonal anti-NXP-2 and immunodepletion experiments*

Preliminary data from Oddis *et al* and Targoff *et al* have described autoantibodies to the MJ antigen, which is a 140-142 kDa protein in JDM (222, 228). The MJ target has recently been identified as nuclear matrix protein NXP-2, also termed MORC-3 (228). Immunodepletion experiments were performed to investigate the identity of the p140 target in this UK JDM cohort, which is likely to be the same as the previously identified MJ autoantigen. Therefore, immunodepletion experiments were performed using different reference anti-p140-JDM-positive sera and a commercial mouse monoclonal anti-NXP-2 antibody. Ten µl of anti-p140 positive sera or 50 µl of commercial mouse antibody to NXP-2 (MORC3) (Medical & Biological Laboratories, Nagoya, Japan) were mixed with 100 µl of prewashed protein G Dynabeads (Dynal, Liverpool, UK) in sodium phosphate (ph 8.1, 0.1 M) at room temperature for 30 min. The antigens were immunoprecipitated as described for IPP, using $[^{35}\text{S}]$ methionine labelled K562 cell extract. Immunodepletion was performed to ascertain whether the
IPP pattern observed with anti-p140 positive JDM sera and the commercial antibody to NXP-2 were due to precipitation of the same antigen. Cell extracts were depleted of autoantibody targets using a reference anti-p140 positive JDM sera and normal serum (NS) as a negative control. In brief, duplicate samples each containing 10 mg protein A sepharose beads (when preparing pre-depleted p140 cell extract for IPP with commercial anti-NXP-2, 150 µl prewashed protein G Dynabeads were used) in 1 ml IPP buffer and 50 µl anti-p140 serum (or 50 µl anti-p155/140 serum) were mixed at room temperature for 30 min. The beads were washed in IPP buffer and 1 tube (A) was placed on ice whilst 120 µl [35S] methionine labelled K562 cell extract and 380 µl IPP buffer was added to tube (B). Tube B was mixed at 4°C for 2 h, the supernatant was then transferred to the corresponding tube A, which was then mixed at 4°C for a further 2 h. The supernatant from the corresponding tubes (A) i.e. p140 antigen depleted cell extract were stored at -80°C. IPP with depleted cell extracts were completed using 50 µl commercial anti-NXP-2, and different anti-p140 sera (10 µl).

**IPP Western blotting with mouse monoclonal anti-NXP-2**

As well as immunodepletion experiments, immunoprecipitation western blotting studies using mouse monoclonal anti-NXP-2 antibodies were also performed. Forty µl of reference sera (anti-p140-JDM sera, anti-p155/140-JDM sera, anti-Mi-2-JDM sera) and 40 µl of normal sera were incubated with washed Sepharose protein A beads. Samples were centrifuged and the supernatant removed. Samples were washed twice with 1 ml triethanolamine and the supernatant removed. Samples were cross-linked and incubated with 1 ml 5mM BS3 in triethanolamine (2.86 mg / ml – 25 mg into 8.74 ml), centrifuged and the supernatant removed. Samples were further incubated with 1 ml 50mM Tris-Cl and then washed three times with PBS and twice with IPP buffer prior to the addition of 1 ml unlabelled K562 cell extract. Samples were mixed at 4°C for 2 hr. Beads were washed in IPP buffer followed by TBS buffer (10 mM Tris-Cl pH 7.4, 150 mM NaCl) before being resuspended in 80 µl SDS sample buffer (Sigma, UK). After heating, proteins were fractionated by 10% SDS PAGE.

Nitrocellulose membrane and blotting paper was soaked in Transfer Buffer (Trizma Base, sodium acetate, EDTA, 0.1% SDS and 20% methanol). A sandwich of blotting paper, gel, nitrocellulose and blotting paper was placed in a transfer cage. Proteins were transferred to nitrocellulose membrane following electrophoresis. The nitrocellulose membrane was washed with Ponceau stain, rinsed with water and air-
dried overnight. The membrane was washed with 0.05% PBS-Tween followed by blocking solution (10% milk powder in PBS-Tween – 10 grm in 100 ml, pH 7.2). The membrane was then incubated with primary antibody (commercial mouse monoclonal anti-NXP-2 antibody – 1:200 dilution – 50 µl in 10 ml block solution) for 1 hr. The membrane was then washed in PBS-Tween, and then incubated with secondary anti-mouse IgG antibody (AbCam, UK), at 1:10,000 dilution in block solution – 50 µl in 50 ml, pH 7.2, for 1 hr. The membrane was washed again with PBS-Tween and water, and then air-dried.

6.2.3 Statistical analysis
The frequencies of clinical features were compared using the Chi-squared test with Yates’ continuity correction or the Fisher’s exact test for groups with small numbers. Where data was not normally distributed the Mann-Whitney-U test was used to compare continuous data. Median values (inter-quartile ranges) were expressed where appropriate and \( P \) values <0.05 were considered significant. \( P \) values (\( P_{corr} \)) were adjusted using the Bonferroni correction. SPSS for Windows (version 14) (for clinical data) was used to perform statistical analysis.

6.3 Results
6.3.1 Anti-p155/140: autoantibodies targeting a 155/140 kDa doublet protein in juvenile dermatomyositis

Serological results
Following IPP, sera from a number of JDM patients recognised two distinct proteins forming a doublet with molecular weights of 155 kDa and 140 kDa (see Figure 16). The same pattern was observed in a subset of adult DM patients (see Chapter 4, Figure 13 and this chapter, Figure 16). Non-specific weak nuclear patterns were observed on IIF between anti-p155/140 patients (data not shown).

The immunodepletion results support the co-identity of the p155/140 kDa doublet precipitated by sera from both JDM and adult DM groups (see Figure 17). When the cell extracts were pre-depleted with normal serum, no targets were removed from the extract and the 155 kDa and 140 kDa autoantigens were still precipitated by juvenile and adult sera. However, when the cell extract was pre-depleted with either juvenile or adult anti-p155/140 positive sera, the autoantigens were no longer detectable in juvenile or adult anti-p155/140 positive sera respectively. This provided
good evidence that the sera from JDM and adult DM contained the same autoantibody specificity.

**Frequency and clinical features of anti-p155/140 autoantibodies**

From 116 juvenile myositis sera, 27 (23%) had anti-p155/140 autoantibodies. Information on the degree of skin involvement and other selected clinical features are outlined in Table 18. There was a higher frequency of males in the anti-p155/140 positive children compared to anti-p155/140 negative children ($P_{corr}=0.04$). Anti-p155/140 positive JDM patients had an increased frequency of skin lesions (Gottron’s papules $P_{corr}=0.027$, ulceration $P_{corr}=0.045$) with a wider distribution of cutaneous involvement, particularly over the small joints ($P_{corr}<0.001$) and large joints ($P_{corr}=0.027$). Cutaneous oedema was also more frequent in anti-p155/140-positive children ($P=0.013$) but this was not significant following multiple corrections. Overall, there was no significant difference in those with elevated muscle enzymes at diagnosis or during disease course or those with an abnormal MRI or muscle biopsy between anti-p155/140 positive and negative groups (data not shown). However not all children had data on this, in particular some did not have a MRI or biopsy performed. There was a trend towards lower CMAS (lower values indicate more severe weakness) and higher PGA in anti-p155/140 positive children at baseline and during follow up, although this did not reach statistical significance. The frequency of other clinical signs including lipoatrophy, arthritis, Raynaud’s, sclerodermatous skin changes, dysphagia, mouth ulcers or alopecia was not significantly different between children with or without anti-p155/140. There was no history of malignancy in the entire JDM cohort during the follow-up period.
**Figure 16:** Immunoprecipitation of p155/140 kDa autoantigens. 10% SDS PAGE of immunoprecipitates of [³⁵S] labelled K562 cell extract. Sera used for immunoprecipitation include Lanes 1-3; adult anti-p155/140 positive serum, Lanes 4-6; juvenile anti-p155/140 positive serum, Lane 9; juvenile anti-Jo-1 (histidyl tRNA synthetase) positive serum, Lane 10; juvenile anti-Mi-2 positive serum. Positions of the p155 and p140 antigens are indicated on the left.
**Figure 17:** Immunodepletion experiments - Autoradiogram of 10% SDS-PAGE of immunoprecipitates using either anti-p155/140-adult DM sera or anti-p155/140-JDM positive sera. Immunoprecipitation was performed with control [$^{35}$S]-methionine-labelled cell extract or [$^{35}$S]-methionine-labelled cell extract depleted with either normal serum (NS), adult positive anti-p155/140 serum or JDM positive anti-p155/140 serum. The bands corresponding to the p155 and p140 autoantigens are indicated.
Table 18: Selected clinical associations of anti-p155/140 autoantibodies in JDM patients

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<thead>
<tr>
<th>Anti-p155/140</th>
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<th>Negative (n=89)</th>
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<td>7 years (5, 10)</td>
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<td>Male:Female</td>
<td>44.4</td>
<td>20.2</td>
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**Type of skin lesion**

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<td>21.3</td>
<td>0.045</td>
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<td>Oedema</td>
<td>63</td>
<td>33.7</td>
<td>NS*</td>
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<td>23.6</td>
<td>NS</td>
</tr>
<tr>
<td>Lipoatrophy</td>
<td>17.9</td>
<td>14.3</td>
<td>NS</td>
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**Distribution of skin lesion**

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<td>92.6</td>
<td>65.9*</td>
<td>NS</td>
</tr>
<tr>
<td>Periungal</td>
<td>81.5</td>
<td>59.1*</td>
<td>NS</td>
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<tr>
<td>Trunk</td>
<td>6 (22.2)</td>
<td>10* (11.4)</td>
<td>NS</td>
</tr>
<tr>
<td>Small joints</td>
<td>100</td>
<td>62.5*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Large joints</td>
<td>77.8</td>
<td>43.2*</td>
<td>0.027</td>
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</table>

**Muscle disease**

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>Baseline CMAS, median (IQR)</td>
<td>36 (13.8, 48)</td>
<td>44 (35, 50.5)</td>
<td>NS</td>
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<tr>
<td>Lowest CMAS, median (IQR)</td>
<td>36 (13.8, 46.8)</td>
<td>43 (29, 49.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Baseline PGA, median (IQR)</td>
<td>4.7 (2.0, 7.1)</td>
<td>2.8 (1.1, 5.0)</td>
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<tr>
<td>Highest PGA, median (IQR)</td>
<td>5.5 (2.5, 7.1)</td>
<td>3 (1.2, 5.1)</td>
<td>NS</td>
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</table>

Values in percentages unless otherwise indicated. *Data on 88 children. **Not all patients had data available for each clinical feature. CMAS (childhood myositis assessment scale: 0-53) at baseline and lowest (worst) score during follow-up. PGA (physician’s global assessment: 0-10) at baseline and highest (worst) score during follow-up.

*$P_{(uncorr)}=0.013$
6.3.2 Anti-p140: autoantibodies targeting a nuclear matrix protein NXP-2 in juvenile dermatomyositis

Serological results

Identification of anti-p140 autoantibodies in JDM sera

Following IPP, sera from a number of JDM patients recognized a distinct protein band with a molecular weight of ~140 kDa (see Figure 18). No anti-p140 positive sera were found to immunoprecipitate any other known MSAs or MAAs. A weak non-specific nuclear pattern or in some cases a negative ANA was observed on IIF in all anti-p140 sera. Anti-p140 was not detected in any of the juvenile disease control sera or healthy adult control sera.

Immunodepletion experiments using anti-140 and anti-p155/140 JDM sera

Immunodepletion confirmed anti-p140 sera target a different protein to JDM sera that recognises the p155/140 doublet protein (see Figure 19, lanes 1-4) showing respective bands are still present following IPP.

The IPP Western blotting with mouse monoclonal anti-NXP-2 studies were negative (did not yield any results). No reference p140 bands were recognised following incubation with primary mouse monoclonal antibody anti-NXP-2 and secondary anti-mouse IgG antibody. This may be because of loss of immunoreactivity of the p140 autoantigen following transfer from SDS-PAGE to nitrocellulose membrane.

Confirmation of the p140 autoantigen

The results suggest that the p140 protein targeted by JDM sera in this study has the same identity as the MJ antigen, NXP-2 (228). Using a commercial antibody raised against NXP-2, IPP resulted in the precipitation of a band with the same molecular weight and IPP pattern as that observed in anti-p140 positive JDM sera (see Figure 20, lane 3). When cell extract was pre-depleted with JDM anti-p140 positive sera, the IPP band present with commercial anti-NXP-2 was no longer detectable (Figure 20, lane 4). The immunodepletion results supported the co-identity of the p140 protein precipitated by different anti-p140 positive juvenile sera. Figure 19 (lanes 5-6) shows an example where the 140 kDa band is no longer detectable following IPP with anti-p140 sera (1) and (2) using pre-depleted reference p140 cell extract.

Clinical associations of anti-p140 autoantibodies

Of 162 juvenile myositis sera serotyped by IPP, 37 (23%) had anti-p140 autoantibodies. This autoantibody specificity was found exclusively in JDM patients,
with a frequency of 27%. Anti-p140 was not detected in any overlap JDM-SSc sera. Selected clinical features of anti-p140 positive patients compared to the overall anti-p140 negative JDM cohort are shown in Table 19. Table 20 shows a comparison between anti-p140, anti-p155/140 and MAA positive cases. There was no significant difference in female:male ratio, age at onset or diagnosis and disease duration (from disease onset to time of this study) between anti-p140 positive and negative cases. Overall there was no significant difference in the presence of Gottron’s lesions, skin ulceration and oedema or the distribution of rash except anti-p140 positive children had no rashes on the trunk compared to anti-p140 negatives ($P_{corr}=0.02$). Anti-p140 positive cases had a significant association with the presence of subcutaneous calcinosis compared to anti-p140 negative patients ($P_{corr}<0.005$, odds ratio 7.0, 95% CI 3.0-16.1). There was no significant difference between other clinical features when comparing anti-p140 positives versus negatives including baseline CK, CMAS, childhood health assessment questionnaire (CHAQ), physician’s global assessment scale (PGA), and the presence of arthritis, Raynaud’s, dysphagia, mouth ulcers and alopecia (data not shown).

Possible clinical differences were noted when anti-p140 positive patients were compared to anti-p155/140 positive patients (Table 18). Overall, age of onset or diagnosis and disease duration (anti-p140 positives, median 48 months IQR 34, 72 versus anti-p155/140 positives, median 52 months, IQR 36, 84) was similar between the autoantibody groups. Compared with anti-p155/140 positive children, anti-p140 positives again had an association with calcinosis, 54% versus 14%, $P_{corr}=0.015$ OR 7.1 (2-25). In contrast, anti-p155/140 positives compared with the anti-p140 positive group had a higher frequency of ulceration and cutaneous oedema however this result was not significant after correcting for multiple comparisons. The distribution of rash was wider on the trunk $P_{corr}<0.005$ and over the small joints ($P_{corr}=0.05$ OR 13.5 95% CI 2-113) in anti-p155/140 compared with anti-p140 positive cases. At the time of diagnosis, anti-p140 compared to anti-p155/140 positive children had a non-significantly lower CK (median 202, IQR 76, 2142 versus median 571 IQR 234, 2495) and LDH (median 845 IQR 710, 1620 versus median 1171 IQR 736, 1647). In addition, anti-p140 positives had a higher CMAS at diagnosis compared to anti-p155/140 positives (median 42, IQR 23, 49 versus median 16, IQR 7, 38) (not significant after adjustment for multiple comparisons). There was a non-significant trend towards a lower baseline CHAQ and PGA in the anti-p140 group compared to the anti-p155/140 group; median CHAQ 1.31 (0.75, 1.63) versus 1.63 (0.78, 2.34), median PGA 5 (3, 7.4) versus 7.3 (5, 7.8) respectively. However, this data needs to
be interpreted in the context that a small number of patients in either group had this data recorded at diagnosis.
Figure 18: Immunoprecipitation of p140 autoantigens
Autoradiogram of 10% SDS PAGE of immunoprecipitates of [35S] labelled K562 cell extract. Sera used for immunoprecipitation include Lane 1; normal serum, Lane 2; anti-p155/140 positive JDM serum, Lanes 3-14; different anti-p140 positive JDM sera.
Figure 19: Immunodepletion experiments with anti-p140 and anti-p155/140

Autoradiogram of 10% SDS-PAGE of immunoprecipitates using different anti-p140 positive JDM sera (lanes 1-2 and 5-6) and anti-p155/140 positive JDM sera (lanes 3-4). Immunoprecipitation was performed with $[^{35}\text{S}]$ labelled K562 cell extract pre-depleted with either reference anti-p140 positive JDM serum or reference anti-p155/140 positive JDM serum.
Figure 20: Immunoprecipitation and immunodepletion of mouse monoclonal nuclear matrix protein NXP-2

Autoradiogram of 10% SDS-PAGE of immunoprecipitates of $^{35}$S labelled K562 cell extract using normal sera (NS) (lane 1), reference anti-p140 positive JDM sera (lane 2) and commercial anti-NXP-2 (lane 3). Lane 4, immunoprecipitation using commercial anti-NXP-2 with $^{35}$S labelled K562 cell extract pre-depleted with reference anti-p140 positive JDM serum.
Table 19: Selected clinical associations of anti-p140 positive JDM patients compared with JDM patients overall (anti-p140 negative) and with anti-p155/140 positive JDM patients *

<table>
<thead>
<tr>
<th></th>
<th>Anti-p140 positive (n=37) **</th>
<th>All JDM anti-p140 negative (n=125)**</th>
<th>Anti-p155/140 positive (n=28)</th>
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<td>(4, 10)</td>
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* Values are the percentage of patients, unless otherwise stated.
** Not all patients had the clinical data available for each feature.

$P_{corr}$: corrected $P$ values, OR: odds ratio (with 95% confidence intervals).
¶ $P_{corr}<0.005$, OR 7.0 (3.0 - 16.1) versus all JDM anti-p140 negative patients and $P_{corr}=0.015$, OR 7.1 (2-25) versus anti-p155/140 positive patients.
# $P_{corr}=0.05$ OR 13.5 (2-113) anti-p155/140 positive patients versus anti-p140 positive patients.
## $P_{corr}=0.02$ versus all JDM anti-p140 negative patients and $P_{corr}<0.005$ versus anti-p155/140 positive patients.
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<tr>
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<th>MAA-positive (n=23) **</th>
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<td>61 ᵉ</td>
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<td>11</td>
<td>48 ᵉ ᵉ</td>
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<td>4 γ</td>
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<tr>
<td>Alopecia</td>
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<td>39</td>
<td>26</td>
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</tbody>
</table>
**Key for Table 20**

* Values are the percentage of patients, unless otherwise stated.

** Not all patients had the clinical data available for each feature.

$P_{corr}$: corrected $P$ values

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>¶</td>
<td>$P_{corr}=0.01$, anti-p155/140-positives versus MAA-positives</td>
</tr>
<tr>
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<td>$P_{corr}=0.03$, anti-p155/140-positives versus MAA-positives</td>
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<tr>
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<td>$P_{corr}=0.02$, anti-p155/140-positives versus MAA-positives</td>
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</tr>
<tr>
<td>#</td>
<td>$P_{corr}=0.045$, anti-p155/140-positives versus MAA-positives / anti-p140-positives</td>
</tr>
<tr>
<td>##</td>
<td>$P_{corr}=0.03$, anti-p155/140-positives versus MAA-positives</td>
</tr>
<tr>
<td>###</td>
<td>$P_{corr}=0.01$, anti-p155/140-positives versus MAA-positives and anti-p140-positives</td>
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<td>γ</td>
<td>$P_{corr}=NS (P=0.04$, anti-p155/140 and anti-p140-positives versus MAA-positives</td>
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6.4 Discussion

As described in this thesis and in previous studies there is now increasing evidence that MSAs are associated with clinical subsets in the adult IIM spectrum. The work described in Chapter 5 and 6 suggests serological profiles may also be associated with clinical phenotypes in JDM. Previously, myositis autoantibodies were detected infrequently in JDM with anti-Mi-2 having the strongest association (see Chapter 5) (125, 127). Therefore, detection and characterisation of novel MSAs in JDM may identify distinct clinical subsets within this disease group. Identification of new autoimmune markers may help clinicians predict clinical outcomes and lead to further insights in disease pathogenesis.

There have been reports of novel protein targets including p140 kDa and p155 kDa polypeptides in both adult and juvenile DM. Two reports have described autoantibodies targeting a 140 kDa protein (anti-MJ) and a 155/(140) kDa protein in US JDM patients (143, 222). In adult DM, anti-p155/140 autoantibodies are associated with a history of malignancy (see Chapter 4) (143, 157, 158). In the study described in Chapter 4 (see Section 4.3.4, Table 12), anti-p155/140 autoantibodies were also detected in adults with CADM with no history of cancer and although the numbers are small, this group of adults tended to be younger. In this cohort of children with JDM, no malignancy has been reported to date in those with anti-p155/140 autoantibodies; long term follow up studies will be required to ascertain whether there is any association with malignancy later in life in this group. In addition, validation of anti-p155/140 in larger groups of both adult and juvenile DM is required. An important finding of this study is the demonstration by immunodepletion that anti-p155/140 autoantibodies appear to target the same autoantigen in both adult and juvenile DM. Targoff et al described anti-p155 (in most cases with a second weaker 140 kDa band) in approximately 30% of their JDM population (143). The clinical specificity of this cohort was not described. This study describes detailed clinical features of JDM patients with autoantibodies targeting p155/140 and confirms they have similar cutaneous clinical associations to adults with the same autoantibody specificity (as described in Chapter 4, Section 4.3.4, Table 11). Like adult DM cases with anti-p155/140 these children have more extensive skin involvement including Gottron's papules over a wider distribution. Anti-p155/140 autoantibodies also appear to define a subset of JDM with more peripheral oedema and skin ulceration. There was a trend towards lower baseline and worst ever CMAS, and higher PGA in anti-p155/140 positive JDM compared to negative patients, although this was not statistically significant. In summary, anti-p155/140
autoantibodies occur frequently in JDM and the preliminary findings suggest that they may identify patients with more severe skin and possibly muscle disease. Identification of this autoantibody at diagnosis may help predict the clinical course.

In this chapter, a further novel autoantibody specificity in the JDM UK cohort is described. Anti-p140 autoantibodies form a major serological subset in juvenile myositis found exclusively in JDM and not detected in any JDM-SSc overlap cases. Anti-p140 positive sera recognised the same polypeptide and did not immunoprecipitate any other known myositis autoantigens, including the anti-p155/140 autoantibody. Combining the data from Study 1 and 2 in this chapter, anti-p140 and anti-p155/140 autoantibodies are detected in approximately 40% of JDM cases, in contrast to a much lower frequency of anti-Mi-2 and other myositis-specific or associated autoantibodies as published previously (10, 125, 127, 219).

The confirmation of a further serological subset in JDM appears to have important clinical implications. Anti-p140 has a significant association with the presence of calcinosis when compared to the overall juvenile myositis cohort. In addition, anti-p140 and anti-p155/140 appear to define JDM into two serological subsets with more homogeneous clinical features. Combined with the data from Study 1 in this chapter, anti-p155/140 positive children appear to have a wider distribution of skin disease, more cutaneous complications including oedema or ulceration and possibly overall higher disease activity but a significantly lower frequency of calcinosis compared to anti-p140 positive children. The clinical differences observed between anti-p140, anti-p155/140 positives and patients without either of these specificities are not explained by differences in time between disease onset or disease duration. This is an interesting observation because factors suggested to influence the development of calcinosis are persistent active disease including chronic cutaneous inflammation (185, 201, 229). The association between serotype and clinical phenotype suggests that the targeted autoantigens p140 and p155/140 may play role in the pathogenesis of skin and soft tissue complications in JDM. Based on previous work that showed an association with the TNFα-308A allele, an increased production of tumour necrosis factor-α and calcinosis (201); future studies to investigate for other potential susceptibility genes including TNF polymorphisms in JDM patients with anti-p140 or anti p155/140 autoantibodies would be of major interest.
The p140 protein targeted by an autoimmune response in our JDM cohort study is consistent with nuclear matrix protein NXP-2, the MJ autoantigen described in preliminary reports in a US JDM cohort, and most recently in an Argentine JDM patient study by Espada et al (222, 225, 228). In the latter study, RNA and protein immunoprecipitation and immunoblotting tested 64 patients with juvenile IIM for autoantibodies. The anti-MJ autoantibody was detected exclusively in 25% of cases, a similar frequency to the anti-p140 autoantibody found in the UK JDM cohort study described here. Clinical features of anti-MJ autoantibodies suggest this specificity is a marker of disease severity, although the numbers were small and no direct comparisons with other serological groups was made. Muscle contractures (44%) and lipoatrophy (44%) were more frequent in anti-MJ-positive cases. This specificity was detected predominantly in pure JDM cases with proximal weakness and classic skin changes. The frequency of calcinosis was 31% and cutaneous vasculitis (with skin ulceration) 38% respectively. Clinical similarities with the anti-MJ and anti-p140 autoantibody are noted between this Argentinean cohort and the UK JDM cohort. The main differences, in particular the prevalence of lipoatrophy, may represent variations in clinical evaluation and length of follow-up for each study (225).

In the study by Espada et al 22% of juvenile patients (all JDM) were positive for the anti-p155/140 autoantibody, again a similar number to that detected in the work in this chapter. All children had proximal muscle weakness with a high prevalence of muscle contracture, and the majority has classic DM skin disease. There was also a relatively high frequency of cutaneous vasculitis with persistent ulceration (57%) (225), the same frequency noted in anti-p155/140-positive children from the UK cohort study (see Table 18 and 19).

A further clinically important complication of JDM is lipodystrophy or lipoatrophy (a loss of subcutaneous fat, which is either localised or generalised affecting the face, trunk and limbs). Previous studies have reported prevalence rates between 12-40% (24, 230, 231). In a recent study, Bingham et al reported a lower frequency of lipodystrophy of 8% in a tertiary referral centre juvenile IIM registry (232). In the Bingham et al study, generalised lipodystrophy was shown to be associated with the anti-p155 (anti-p155/140) autoantibody (143), as well a chronic disease course, muscle atrophy, joint contractures, and interestingly calcinosis (232). In this UK cohort study, the overall frequency of lipodystrophy/atrophy was 14% (see Chapter 5, Section 5.3.2, Table 16), although the prevalence of this complication in anti-p155/140 and anti-p140 positive children was not significantly different compared to
the rest of the cohort. Interestingly, lipoatrophy was seen in a relatively higher frequency of children who were positive for MAAs, however this did not reach statistical significance. A potential reason for the difference in lipoatrophy associations between this study and the work by Bingham et al (232) is the way respective clinicians define lipodystrophy or lipoatrophy. Furthermore, the relatively higher frequency of lipoatrophy in MAA-positive children may be because this overall autoantibody group was associated with JDM-scleroderma overlap.

Larger collaborative studies are required to evaluate further the clinical specificity of both anti-p155/140 and anti-p140 autoantibodies in juvenile myositis populations. Work to date and the work presented in this chapter suggest that these two specificities are very important autoantibody systems with pathogenic, clinical and prognostic implications. When grouped together these two serological subsets are positive in between 40-50% of JDM cases, and appear to define those children with a more severe disease course including an association with widespread skin lesions, skin ulceration, oedema, lipodystrophy, calcinosis and worse muscle disease with muscle contracture (222, 225, 232, 233) (and Chapter 6).

The identity of the p155 kDa and p140 kDa doublet protein requires investigation. It is likely that the p155/140 autoantigen identified in this work is the same as the p155/140 doublet proteins identified in recent studies (143, 157, 158), although immunodepletion or reverse IPP blotting studies with prototype sera are required for further confirmation. A preliminary report has identified the target of the anti-p155 autoantibodies as transcriptional intermediary factor 1-gamma (TIF1-γ) (159). This nuclear protein is a member of a novel family of transcriptional coregulator-encoding genes, which function in cell differentiation and development (181). As yet, it is unclear what relationship the p140 protein (as part of the p155/140 doublet) has to the identified p155 TIF1-γ autoantigen i.e. is this a separate protein, which forms part of a multi-protein complex, or is it simply a cleaved or modified fragment of the same protein.

Augmented expression myositis-specific autoantigens in diseased muscle and cancer tissue suggests a new paradigm for pathogenesis of IIM (107, 137). It is not clear why a tumour-associated autoantigen in adult DM should be recognised in JDM, particularly because there appears to be no association malignancy. However, other clinical features including the degree of skin disease in anti-p155/140 positive children and adults are very similar. Longitudinal prospective studies are required to
investigate whether this autoantibody in juvenile disease persists into adulthood and if so are they clinically significant. Perhaps the observation that the same autoantigen system is expressed in younger DM adults without cancer as well JDM suggests that some perturbation of p155/140 expression in proliferating cells combined with a more efficient anti-cancer response by a younger immune system may be a unifying mechanism to explain why it is targeted by an autoimmune response in children and adults with DM.

The p140 autoantigen NXP-2 has nuclear matrix binding, RNA-binding, and coiled-coil domains that are structurally separated, which implicates a role in diverse nuclear functions including regulation of transcription and RNA metabolism (228, 234). The other dermatomyositis-specific autoantigen targets in children and adults; Mi-2 and p155/140 (p155 – transcription intermediary factor 1-gamma) (159, 181) are nuclear proteins that also mediate gene transcription. It is of further interest to note that autoantibodies to small ubiquitin-like modifier enzyme (SAE), which is involved in post-transcriptional modification termed sumoylation, have been described in adult dermatomyositis (see Chapter 4, Section 4.3.2) (160). It is interesting that anti-SAE autoantibodies were not detected in any sera from the UK JDM cohort (Chapters 5 and 6), particularly because NXP-2 has been shown to be a sumoylation target involved in transcriptional repression (235). However, this observation may suggest shared pathogenic mechanisms in both juvenile and adult dermatomyositis.

In conclusion, anti-p155/140 and anti-p140 are clinically important serological markers found in a high frequency of JDM patients that are associated several disease complications, which confers significant morbidity. In the future, routine testing of these novel autoantibodies at disease onset could have prognostic value and identify those children at risk of more severe disease, which may influence management. Furthermore, increasing our understanding of autoimmune targets and their relationship to clinical phenotype in juvenile myositis may provide further insight into pathogenic pathways, which in turn will stimulate new therapeutic approaches.
6.5 Appendix

I. Acknowledgements

I would like to thank Mrs J Dunphy and Mrs P Owen for their assistance and guidance in performing ANA IIF on the juvenile myositis samples. Thank you to Dr Zoe Betteridge for her guidance with my immunoprecipitation, immunodepletion and western blotting studies. I would also like to thank Prof Lucy Wedderburn, Scientific Director for the Juvenile Dermatomyositis UK Cohort Study for her collaboration, guidance and support.

On behalf of the Juvenile Dermatomyositis Research Group I would like to thank all the patients and their families who contributed to the Juvenile Dermatomyositis Cohort Study. I thank all local research coordinators and principal investigators who have made this research not have been possible. The members who contributed were: Mr Ian Roberts, The Royal Liverpool Children’s Hospital, Alder Hey, Liverpool, and Booth Hall Children’s Hospital, Manchester; Dr Eileen Baildam, Booth Hall Children’s Hospital, Manchester and now Alder Hey, Liverpool; Dr Phil Riley, Booth Hall Children’s Hospital, Manchester; Mrs Janis Scott and Dr Clive Ryder, Birmingham Children’s Hospital, Birmingham; Mrs Gillian Jackson and Dr Sue Wyatt, Leeds General Infirmary, Leeds; Ms Elizabeth Camp and Dr Janet Gardner-Medwin, The Royal Hospital for Sick Children, Yorkhill, Glasgow; Mrs Alison Swift, Dr Helen Foster and Dr Mark Friswell, The Royal Victoria Infirmary, Newcastle; Mrs Elizabeth Hutchinson and Dr Helen Venning, Queens Medical Centre, Nottingham and Dr Clarissa Pilkington, Dr N Hasson and Ms Sue Maillard, Great Ormond Street Hospital, London. I thank H Varsani for technical assistance, in particular preparation of the serum samples, which were sent to the RNHRD for this study. The Arthritis Research Campaign awarded me the Barbara Ansell fellowship in Paediatric Rheumatology (grant 18136) to perform this work. I am grateful to the Arthritis Research Campaign who funded the autoantibody testing for this study as part of my fellowship.
CHAPTER SEVEN
DISCUSSION

Introduction
This chapter will outline two preliminary studies conducted alongside the main body of this thesis. The chapter will close with a general discussion of the work conducted as part of this thesis, and its relevance to the current understanding of the relationship between pathogenesis, serological subsets and clinical phenotypes in adult and juvenile IIM. To conclude, hypothetical models of autoimmune dysregulation in distinct microenvironments and how this may lead to disease initiation and propagation in IIM, will be explored. Potential future work focusing on antigen expression at a cellular and tissue level, and the development of solid phase assays for commercial testing of novel myositis-specific autoantibodies (MSAs), will be discussed.

7.1 Other preliminary studies
7.1.1 Autoantibodies in idiopathic interstitial pneumonia

Introduction
As described in previous chapters, interstitial pneumonia is a frequent and significant manifestation of adult IIM, particularly the anti-synthetase syndrome (ASS) subset. Moreover, interstitial lung disease may be the presenting or predominant manifestation of IIM, especially in ASS "sine myositis" and clinically-amyopathic DM (29, 31, 40-42).

NSIP has been reported to be the most frequent subtype on both HRCT and lung biopsy in IIM (38). AIP, OP and UIP have also been reported and identified as patterns responsible for acute or rapidly progressive interstitial pneumonia with AIP and UIP associated with the worst prognosis (28-31, 33, 39). It is well recognised that interstitial lung involvement may be the first or predominant organ manifestation preceding muscle or skin disease, particularly in the anti-synthetase syndrome (see Section 1.5.1) and in patients with clinically-amyopathic DM (CADM) (see Section 1.5.3). Similarly, it is now recognised that interstitial pneumonia can be the major complication of systemic sclerosis sine scleroderma, particularly in association with the specific scleroderma-associated autoantibody anti-Th/To (227, 236-238).

Diffuse parenchymal lung disease is labelled idiopathic interstitial pneumonia when there is no evidence of a causative agent or coexisting connective tissue disease. The standard autoimmune profile is usually negative, even in patients who later
manifest a connective tissue disease (CTD). Therefore some patients with interstitial pneumonia may have underlying CTD characterised by the presence of specific autoantibodies not detected on routine serological testing, particularly ANA ELISA (most commonly used in routine clinical laboratories). In this pilot study, a small cohort of patients previously diagnosed as having idiopathic interstitial pneumonia have been screened for CTD-associated autoantibodies using ANA IIF and protein immunoprecipitation (IPP).

**Patients and Methods**

Twenty patients with a previous clinico-radiological diagnosis of idiopathic interstitial pneumonia were studied (patients from the Royal United Hospital Interstitial Lung disease clinic were invited to participate and not pre-selected). On further detailed clinical review by a Rheumatologist (HG), some patients had clinical features suggestive of an underlying CTD including a history of Raynaud’s phenomenon (see Table 22).

All patients had high-resolution computerised tomography (HRCT) scans, which were re-reviewed (see Section 3.2.2). The subtype of interstitial pneumonia was categorised based on the American Thoracic Society / European Respiratory Society classification (32).

Patients’ sera were analysed using ANA indirect immunofluorescence (IIF) and further characterised using protein immunoprecipitation of $^{35}$S-labelled K562 cells combined with SDS-PAGE to identify specific autoantigen targets, as described previously (see Chapter 2, Section 2.3.1 and 2.3.2).

**Results**

For detailed results see Table 21 and see Figure 21. Six patients had a positive ANA on IIF (>1/40 titre), all with a homogeneous pattern. Of those, two patients’ sera were negative on IPP, three cases had strong unidentified bands, and one patient was anti-topoisomerase positive on IPP (characteristic 100 kDa band, and confirmed by ENA ELISA). Despite two patients testing negative on ANA IIF, both had unidentified bands on IPP.

Five patients’ sera with negative anti-nuclear staining but a strong cytoplasmic speckle tested positive for recognised anti-aminocyl tRNA synthetase (ARS)
autoantibodies (one anti-Jo-1, one anti-PL-7, two anti-PL-12, and one patient with possible anti-Ha autoantibodies). See Table 22.

**Discussion**

In this preliminary study, 30% of patients with a previous diagnosis of idiopathic interstitial pneumonia actually tested positive for CTD-associated autoantibodies. One case tested positive for anti-topoisomerase autoantibodies, with NSIP lung disease, and clinical features of Raynaud’s phenomenon. Therefore, the patient may be re-diagnosed as having systemic sclerosis sine scleroderma (major SSc organ involvement, in this case fibrotic lung disease, a positive SSc-autoantibody, without characteristic skin changes of scleroderma) (239). In two recent studies by Fischer et al, a subset of patients with idiopathic interstitial pneumonia had nucleolar staining patterns of ANA IIF, the majority of which were positive for anti-Th/To autoantibodies (236, 237). Anti-Th/To is associated with less sclerodermatous features and a higher risk of pulmonary hypertension and lung fibrosis (227, 239), and this autoantibody specificity should be considered in patients with these clinical manifestations.

Of those 30% with CTD-autoantibodies, the other cases were positive anti-ARS autoantibodies, in particular non-Jo-1-anti-ARS. All non-Jo-1-anti-ARS has a clinicoradiological pattern consistent with UIP, except the anti-PL-7 case, which had NSIP. As described in Chapter 3 (see Section 3.3.3), lung fibrosis is often the predominant clinical manifestation in non-Jo-1-ARS-positive patients. Furthermore, radiological patterns demonstrate more architectural distortion with pronounced traction bronchiectasis and honeycombing, and histologically fibroblastic foci. This subtype of interstitial pneumonia is synonymous with idiopathic pulmonary fibrosis (formerly termed cryptogenic fibrosing alveolitis). Therefore, it is possible a proportion of patients with idiopathic pulmonary fibrosis actually have non-Jo-1-ARS, especially anti-PL-12 autoantibodies (42, 150). Anti-PL-7 autoantibodies have also been shown to be significantly associated with interstitial pneumonia and much milder or subclinical muscle disease (145).

In summary, patients with a presumed idiopathic interstitial pneumonia may have subtle features of underlying CTD, and a negative routine autoimmune panel should not dissuade clinicians to consider more novel serological markers of autoimmunity in some. Moreover, even in patients with UIP lung disease, a negative ANA but a strong cytoplasmic pattern on IIF should prompt more detailed investigation. A
proportion of these patients may be a “formes fruste” of an underlying CTD, in particular anti-synthetase syndrome where lung disease is the only or predominant manifestation. Historically, the general consensus has been patients with idiopathic lung fibrosis have a limited response to immunomodulatory therapy, whereas patients with CTD-associated lung disease do appear to respond or stabilise with corticosteroid and immunosuppressive therapies. Thus, early identification of this group of patients will influence treatment strategies and long-term prognosis.

Part of this preliminary study has been published in abstract form:
Table 21: Serological results in idiopathic interstitial pneumonia patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>IIP subset</th>
<th>ANA on IIF</th>
<th>IPP</th>
</tr>
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<tbody>
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<td>R23840</td>
<td>61</td>
<td>NSIP</td>
<td>1/160 homogeneous</td>
<td>Negative</td>
</tr>
<tr>
<td>R23839</td>
<td>73</td>
<td>NSIP</td>
<td>Negative</td>
<td>Non-specific bands,</td>
</tr>
<tr>
<td>R23838</td>
<td>74</td>
<td>NSIP</td>
<td>Negative with strong</td>
<td>Anti-Jo-1</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>cytoplasmic speckle</td>
<td></td>
</tr>
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<td>UIP</td>
<td>1/40 homogeneous</td>
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</tr>
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<td>Negative</td>
<td>Negative</td>
</tr>
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<td>Negative</td>
<td>Negative</td>
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<td>NSIP</td>
<td>Negative but strong</td>
<td>Anti-PL-7</td>
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<td></td>
<td></td>
<td>cytoplasmic speckle</td>
<td></td>
</tr>
<tr>
<td>R24102</td>
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<td>RBILD</td>
<td>1/40 homogeneous and</td>
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<td></td>
<td></td>
<td>fine cytoplasmic speckle</td>
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</tr>
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<td>Negative</td>
<td>Negative</td>
</tr>
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<td>Anti-PL-12</td>
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<td></td>
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</tr>
</tbody>
</table>

RP: Raynaud’s phenomenon  
MH: Mechanic’s hands  
Topo: Topoisomerase
Figure 21: Immunoprecipitation study in idiopathic lung disease patients

21A

Autoradiogram of 10% SDS-PAGE of immunoprecipitates of $[^{35}\text{S}]$ labelled K562 cell extract using normal serum (lane 1), reference anti-PL-12 and reference anti-PL-7 sera (lane 2), reference anti-Jo-1, reference anti-U1-RNP and reference anti-RNAP-II sera (lane 3), reference anti-PM-Scl, reference anti-Ro and reference anti-La sera (lane 4), R23840 serum (lane 5), R23839 serum (lane 6), R23838 (anti-Jo-1) serum (lane 7), R23837 serum (lane 8), R23843 serum (lane 9), R23842 serum (lane 10), R24103 serum (lane 11), and R24101 (anti-PL-7) serum (lane 12 – arrow).
Figure 21: Immunoprecipitation study in idiopathic lung disease patients

21B

Autoradiogram of 10% SDS-PAGE of immunoprecipitates of $[^{35}\text{S}]$ labelled K562 cell extract using reference anti-U3-RNP serum (lane 1), R2518 serum (anti-Topoisomerase) (lane 2), reference anti-mitochondrial serum (lane 3), reference anti-Ku serum (lane 4), R24102 serum (lane 5), R24100 serum (lane 6), R19803 serum (lane 7), R19338 serum (lane 8), control normal serum (lane 9), R24526 serum (lane 10), and R25133 serum (lane 11). Arrow – 100 kDa.
Figure 21: Immunoprecipitation study in idiopathic lung disease patients
21C

7.1.2 Anti-p140 autoantibodies in adult IIM

Introduction
As described in Chapter 6, a major autoimmune target in JDM is the p140 autoantigen, likely to be the nuclear matrix protein NXP-2. Following detailed serological analysis of adult IIM patients recruited to the RNHRD cohort study and AOMIC UK Registry (work described in Chapters 3 and 4), a subset were noted to also immunoprecipitate a p140 kDa band. Therefore, preliminary work was undertaken to investigate if autoantibodies to the same p140 autoantigen, are present in adult myositis sera.

Patients and Methods
Clinical data and serum samples were available from the UK Adult Onset Myositis Immunogenetic Collaboration (AOMIC) and the RNHRD IIM cohort study (as described in Chapters 2-4). As previously described, serum samples were autoantibody typed by immunoprecipitation using $^{35}$S labelled K562 cells. Immunofluorescence using HEp-2 cells and fluorescein-labelled anti-human immunoglobulin was completed on adult anti-p140 positive samples.

Results
Sera from 266 myositis patients were screened for anti-p140 autoantibodies. Overall, seven cases (3%) were positive for anti-p140 autoantibodies, which were detected exclusively in 5% of DM patients. From the RNHRD cohort study, two reference sera (R18883 and R23389) were positive for autoantibodies to a p140 kDa band. A further five patients’ sera were found to recognise a 140 kDa band on SDS-PAGE. No anti-p140 antibody positive patients were positive for other recognized autoantibodies (see Figure 22). IPP experiments using cell extract pre-depleted with reference adult-p140-positive sera (R18883) suggest that the identity of the p140 target in adult DM may be the same as the p140 protein recognised in JDM, and different to the p155/140 autoantigen (see Figure 23). ANA IIF results are shown in Table 23. The major clinical features of anti-p140-positive patients were heliotrope rash (73%), Gottron’s lesions (82%), periungal erythema (91%) and systemic involvement including weight loss or fever (78%). In particular, five out of seven (71%) anti-p140-positive adult patients had interstitial pneumonia. There was no cancer-associated myositis in the adult anti-p140 positive subset. In contrast to anti-p140-positive JDM patients where calcinosis is a significant feature, this was only present in one patient.
Dr Zoe Betteridge (and HG) have since conducted further work. This work has been published in abstract form:


In total, serum and clinical data has now been analysed in 443 adults with myositis (PM and DM), based on the Bohan and Peter diagnostic criteria, recruited to the Adult Onset Myositis Immunogenetic Collaboration, UK, RNHRD Cohort and the Institute of Rheumatology, Prague, CZ. All sera were autoantibody typed by immunoprecipitation using $^{35}$S-labelled K562 cells. Immunofluorescence using Hep-2 cells and fluorescein-labelled anti-human immunoglobulin was completed on p140 positive samples.

Thirteen (2.93%) adult patients with IIM were positive for anti-p140 autoantibodies. All anti-p140 positive patients had DM. The prevalence of anti-p140 autoantibodies specifically in the DM cohort was 5.86%. Anti-p140 autoantibodies were not detected in sera from normal healthy controls or PM, myositis-overlap, SSc or SLE patients.

The major clinical associations of anti-p140 autoantibodies in adults are shown in Table 24. In particular, the frequency of interstitial pneumonia in anti-p140 positive patients was 61.5% in comparison to 26.2% in the DM anti-p140 negative group (p=0.016). In addition, no p140 positive patients had cancer-associated myositis and in comparison with the JDM cohort, where calcinosis was a significant association, calcinosis was only present in two adult p140-positive patients (15.4%).

**Discussion**

Anti-p140 autoantibodies form a further serological subset in adult DM. In addition, preliminary data suggests that the clinical associations of anti-p140 autoantibodies in adults differ from JDM. In particular, lung disease appears to be a major feature of anti-p140-positive adult DM associated with hallmark cutaneous disease. Screening of larger IIM cohorts will further characterise the anti-p140 clinical phenotype in comparison to other IIM serological subsets, specifically novel specificities anti-SAE and anti-p155/140.
Figure 22: Immunoprecipitation of anti-p140 autoantibodies in adult DM

Figure 23: Immunodepletion experiments with reference anti-p140-positive JDM and adult DM sera, and anti-p155/140-positive JDM sera

Table 23: Anti-p140 autoantibody ANA IIF patterns (adult DM)

<table>
<thead>
<tr>
<th>Sample</th>
<th>ANA IIF pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>R18883</td>
<td>Fine speckle nucleolar sparing and fine cytoplasmic speckle</td>
</tr>
<tr>
<td>R23389</td>
<td>Fine speckle nucleolar sparing 1/40</td>
</tr>
<tr>
<td>M89</td>
<td>Homogeneous</td>
</tr>
<tr>
<td>M136</td>
<td>Fine speckle nucleolar sparing</td>
</tr>
<tr>
<td>M145</td>
<td>Homogeneous</td>
</tr>
<tr>
<td>M203</td>
<td>Fine speckle nucleolar sparing 1/40</td>
</tr>
<tr>
<td>M212</td>
<td>Homogeneous</td>
</tr>
</tbody>
</table>

Table 24: Clinical Features of anti-p140 autoantibodies in adult dermatomyositis

<table>
<thead>
<tr>
<th>Clinical Feature</th>
<th>Anti-p140 autoantibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (n=13)</td>
</tr>
<tr>
<td>Gender</td>
<td>53.8% Female</td>
</tr>
<tr>
<td>Gottron's Lesions</td>
<td>92.3%</td>
</tr>
<tr>
<td>Heliotrope Rash</td>
<td>76.9%</td>
</tr>
<tr>
<td>Raised Creatinine Kinase</td>
<td>53.9%</td>
</tr>
<tr>
<td>Systemic Features</td>
<td>76.9%</td>
</tr>
<tr>
<td>Cancer</td>
<td>0%</td>
</tr>
<tr>
<td>Interstitial pneumonia</td>
<td>61.5% *</td>
</tr>
<tr>
<td>Weakness</td>
<td>69.2%</td>
</tr>
</tbody>
</table>

*P_{uncor}=0.016 (OR 4.5 95% CI: 1.4-14.4)
7.2 Summary and Final Discussion

7.2.1 Myositis-specific autoantibodies

The work described in this thesis combined with various studies over the past few years provides evidence highlighting the importance of autoimmunity in myositis. Up to 80% adult patients and 60% of juvenile patients now have well defined MSAs and MAAs when tested by detailed serological techniques.

- Eight anti-synthetase autoantibodies collectively define the anti-synthetase syndrome, characterised by myositis, skin disease including mechanic’s hands and Gottron’s papules, non-erosive arthritis, fever, Raynaud’s and interstitial pneumonia. Lung involvement is a major complication and may be the predominant clinical manifestation.
- Anti-SRP autoantibodies define patients severe acute / subacute necrotising myopathy, which may be refractory to standard treatment.
- Anti-Mi-2 autoantibodies define patients with classic hallmark DM who appear to respond well to standard treatments and run a monocyclic disease course.
- Anti-CADM-140 (MDA5) autoantibodies appear to be unique to Asian clinically-amyopathic DM patients with a high risk of progressive acute interstitial pneumonia.
- Anti-p155/140 is a novel autoantibody found in both adult DM and JDM. This specificity defines patients with severe cutaneous disease, and is associated with cancer-associated myositis in older adults.
- Anti-SAE autoantibodies are detected in adult DM who may present with clinically-amyopathic DM first, progressing to myositis and systemic features. Interstitial lung involvement appears infrequent.
- Anti-p140 autoantibodies are a major serological subset in JDM, and are associated with calcinosis.

The identification and characterisation of an increasing number of MSAs and their corresponding targets has emphasised how we should now consider a clinico-serological classification in IIM, rather than simply diagnose patients with PM, DM or CTD-myositis overlap. In the future, if MSAs are used to define patients into clinical syndromes this may help predict outcomes and thus influence treatment strategies.
<table>
<thead>
<tr>
<th>Autoantibodies</th>
<th>Target autoantigen and function</th>
<th>Clinical Phenotype</th>
<th>Autoantibody frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Adults</td>
</tr>
<tr>
<td>Anti-ARS</td>
<td>ARS – intracytoplasmic protein synthesis</td>
<td>Anti-synthetase syndrome</td>
<td>30-40%</td>
</tr>
<tr>
<td>Anti-Jo-1</td>
<td>Histidyl</td>
<td>Myositis, mechanic’s hands, Gottron’s papules, arthritis, fever, Raynaud’s phenomenon, high frequency of interstitial pneumonia</td>
<td></td>
</tr>
<tr>
<td>Anti-PL-7</td>
<td>Threonyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-PL-12</td>
<td>Alanyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-EJ</td>
<td>Glycyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-OJ</td>
<td>Isoleucyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-KS</td>
<td>Asparaginyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Ha</td>
<td>Tyrosyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Zo</td>
<td>Phenylalanyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-SRP</td>
<td>SRP – intracytoplasmic protein translocation (6 polypeptides and ribonucleoprotein 7SLRNA)</td>
<td>Acute onset necrotizing myopathy (severe weakness, high CK) May be refractory to treatment</td>
<td>5%</td>
</tr>
<tr>
<td>Anti-Mi-2</td>
<td>Helicase protein – nuclear transcription (Forms the NuRD complex)</td>
<td>Adult DM and JDM (hallmark cutaneous disease, milder muscle disease with good response to treatment)</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>Anti-p155/140</td>
<td>TIF1-γ (p155) – nuclear transcription + cellular differentiation</td>
<td>CAM in adult DM Severe cutaneous disease in adult DM and JDM</td>
<td>13-21%</td>
</tr>
<tr>
<td>Anti-p140</td>
<td>Likely to be NXP-2 – nuclear transcription + RNA metabolism</td>
<td>JDM with calcinosis</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-SAE</td>
<td>SAE - post-translational modification (targets include transcription factors)</td>
<td>Adult DM May present with CADM first</td>
<td>5%</td>
</tr>
<tr>
<td>Anti-CADM-140</td>
<td>Intracytoplasmic MDA5 – innate immune responses against viral infections</td>
<td>CADM Rapidly progressive interstitial pneumonia</td>
<td>Overall - unknown</td>
</tr>
</tbody>
</table>
Figure 24: Summary: Myositis-specific autoantibodies and clinical features
7.2.2 Pathogenic mechanisms: MSAs and autoantigens as clues

There is strong evidence that autoimmune mechanisms play an important role in both polymyositis (PM) and dermatomyositis (DM). Previous studies have reported that around 40-50% of adults and 20-30% of juvenile IIM patients have the presence of high-titre autoantibodies. Myositis autoantibodies can be categorised into myositis associated autoantibodies (MAAs) and myositis specific autoantibodies (MSAs). The MAAs; anti-U1-RNP, anti-U3-RNP (fibrillarin), anti-PM-Scl and anti-Ku are principally seen in myositis-scleroderma overlap syndromes (240), whereas the MSAs are highly selective, mutually exclusive and are associated with particular genotypes and clinical phenotypes within the myositis spectrum (6, 8, 9, 241). This thesis describes a comprehensive, clinical and serological study of adult and juvenile idiopathic inflammatory myopathy. Based on this body of work combined with additional preliminary studies, the frequency of MSAs/MAAs is nearer 80% in adults and 60% in children, therefore dispelling the notion that the majority of myositis cases are 'seronegative' (241-243).

MSAs are directed against both cytoplasmic and nuclear components of the cell involved in key regulatory intra-cellular processes, including gene transcription, protein translocation and anti-viral responses. Interestingly, autoantigens with analogous cellular functions are associated with similar clinical subsets. For example, as described in previous chapters, the anti-ARS autoantibodies target functionally related cytoplasmic tRNA enzymes involved in protein synthesis. This group of autoantigens form the largest subset recognised in adult IIM patients, and are associated with distinct clinical features collectively termed the anti-synthetase syndrome. A further subset of myositis patients with severe necrotising myopathy is characterized by the presence of autoantibodies directed against the signal recognition particle (SRP) (121, 122). SRP is a cytoplasmic ribonucleoprotein complex that recognizes secreted and membrane-bound proteins regulating protein translocation through the endoplasmic reticulum.

In contrast, autoantibodies associated with DM clinical phenotypes, such as anti-Mi-2 and novel MSAs anti-p155/140 and anti-p140, target nuclear proteins (126, 143, 157, 158, 161). Mi-2 is a nuclear helicase protein that forms part of the nucleosome remodelling deacetylase (NuRD) complex playing a role in gene transcription, specifically chromatin remodelling (129). Targoff et al has identified the p155 target of the p155/140 complex as transcriptional intermediary factor 1-gamma (TIF1-γ), a nuclear protein involved in cellular differentiation (159). In addition, the p140 target
has been identified as nuclear matrix protein NXP-2, which plays a role in RNA metabolism and maintenance of nuclear architecture (228, 234). Finally, anti-SAE autoantibodies target the small ubiquitin-like modifier activating enzyme subunits involved in post-translational modification that is located in both the nucleus, but unlike other DM-associated autoantigens, it is also found in the cytoplasm of cells (168). The potential relationship between the DM-specific autoantigens is thus of interest. It is possible SAE plays a central role forming stable conjugates with other proteins including transcription factors (168). Interestingly, a specific target of sumoylation is a protein (p66) that has been shown to mediate transcriptional repression of the Mi-2/NuRD complex (178). Furthermore, NXP-2 has been reported to have a role in sumoylation-mediated regulation of transcription (235). Post-translational modification can lead to the generation of self-antigens and so this observation suggests shared pathogenic mechanisms.

The exception to the above observations may be the anti-CADM-140 autoantibody, now described in two Asian DM cohorts with CADM and severe lung involvement (130, 131, 244). The identity of this cytoplasmic protein CADM-140 has recently been discovered to be melanoma-differentiation-associated gene 5 (MDA5) (131, 244). The MDA5 autoantigen appears to be distinct in terms of its biological function and disease expression. MDA5 is involved in the innate immune defence against viral infections through the detection of viral dsDNA (132). This observation may be explained by the fact that anti-CADM-140-autoantibody DM appears to be a unique clinical subset found only in Asian ethnicity, with specific genetic and environmental determinants.

In general, the systemic autoimmune diseases are a genetically complex heterogeneous group of diseases in which the immune system attacks diverse but highly specific intracellular self-structures. In IIM, the striking association between specific autoantigen targets and clinical phenotypes, to the degree that IIM can now be classified into more homogeneous subsets, suggests that the autoantigen itself may be central in determining disease expression. Moreover, in IIM there are key target tissues, in particular the muscle, the skin, the lungs, and in some cases tumour tissue. It is possible that in IIM syndromes, disease initiation is dependent on the structure and concentration of autoantigens (i.e. novel non-tolerized forms – truly novel epitopes or previously recognised but modified epitopes that lower the threshold for autoreactive T cells to be activated) in certain localised pro-immune microenvironments. Several studies have provided insight not only into the potential
importance of the autoantigen target but also it’s expression in specific microenvironments. Two studies have highlighted how certain autoantigenic -tRNA synthetases (histidyl, asparaginyl and tyrosyl) have chemoattractant properties and can induce leukocyte migration via the CCR5 and CCR3 receptors respectively (135, 245). In comparison, non-antigenic -tRNA synthetases (aspartyl and lysyl) do not activate chemokine receptors. In addition, mononuclear cells expressing chemokine receptors are present in myositis muscle but not normal muscle (135). The authors suggested that autoantigenic ARS are over-expressed in damaged muscle cells and their pro-inflammatory properties promote the immune response, which leads to the development of myositis.

Several studies have now demonstrated that certain autoantigens are enriched in lesional tissue involved in IIM. In the seminal paper by Casciola-Rosen et al several myositis-specific and associated autoantigens (including the Jo-1 and Mi-2 proteins were shown to be upregulated in myositis muscle in comparison to normal muscle especially in regenerating muscle cells, rather than mature myotubes (107). Zampieri et al demonstrated similar findings in newborn skeletal muscle, in the absence of infiltrating inflammatory cells. Using immunocolocalisation techniques, anti-Jo-1 sera showed a positive reaction within the cytoplasm of tibialis anterior muscles (TA) from newborn rat cyrosections and anti-Mi-2 positive serum gave a homogenous staining of myonuclei. In contrast, sections of TA from adult rats gave negative staining with both anti-Jo-1 and anti-Mi-2 sera. In addition, staining of Jo-1 and Mi-2 paralleled the staining with anti-MHCemb, further indicating that expression of Jo-1 and Mi-2 is enhanced in myotubes (246). Collectively, these findings support the hypothesis that the presence of candidate myositis autoantigens during reparative myogenesis can drive induction and propagation of the autoimmune response.

It has been proposed that susceptibility to the serine protease, granzyme B, is strongly predictive of autoantigen status. Casciola-Rosen et al have shown that isoleucyl-, histidyl- and alanyl-tRNA synthetases, Mi-2 and SRP72 are all cleaved by granzyme B, leading to the release of unique fragments that contain autoantigenic epitopes (104). Work by Levine et al, has emphasized the potential pathogenic role of proteolytic cleavage of autoantigens in lesional tissues. They demonstrated that the histidyl-tRNA protein exists in two conformations and whilst there are similar overall expression levels of Jo-1 in different tissues, the granzyme B cleavable form is enriched in the alveolar epithelial layer of lung (108). Therefore, the theory that
distinct microenvironments may shape disease expression was emphasised with this finding that a novel conformation of Jo-1 is enriched in the lung in comparison to other tissues including muscle. This has lead to the suggestion that the initiating target tissue for the autoimmune response in the anti-Jo-1 syndrome is the lung with secondary attack to muscle.

Further evidence implicating autoantigen driven responses was suggested in a study by Katsumata et al (133). They generated an antigen-induced model of IIM following immunisation of congenic mice with either human or murine Jo-1 protein or amino terminal peptides of Jo-1 protein. Early antibody responses demonstrated strong species-specificity with limited cross recognition of Jo-1 between species. Studies on serum samples 8 weeks after immunisation revealed epitope spreading with the breakdown of B-cell tolerance to ubiquitously expressed native target autoantigens. The presence of anti-Jo-1 IgG isotypes indicated class switching and T-cell dependent B-cell responses, likely reflecting a breakdown in T-cell tolerance to self Jo-1. Assessments of mice after murine Jo-1 immunization demonstrated the presence of skeletal muscle inflammation and interstitial lung disease, which are clinical characteristics of the ASS clinical phenotype in humans.

Work on the Mi-2 protein gives further emphasis to the potential role of target proteins in disease expression. Casciola-Rosen et al have previously demonstrated that Mi-2 is preferentially expressed in DM muscle rather than PM muscle, supporting the association between DM and Mi-2 (107). Using a conditional gene targeting approach in a mouse model, Kashiwagi et al demonstrated that Mi-2 is essential for the development and repair of the basal epidermis (134). Furthermore, studies by Burd et al have reported that following UV radiation, Mi-2 protein levels in a human keratinocyte cell line are rapidly up-regulated and maintained by an increase in translation efficiency through a regulatory element in the 5'-UTR region of mRNA and increased protein stability. This suggests enhanced expression of Mi-2 in UV exposed keratinocytes leads to the initiation of autoimmunity and subsequently the DM phenotype associated with classic skin lesions. Autoantibodies to other members of the NURD complex have not been reported in IIM, and the effects of UV radiation do not significantly affect other NURD proteins, leading to the hypothesis that Mi-2 may act independently of the NURD complex in response to DNA damage (247).
The association between malignancy and myositis, particularly in adult DM is well recognised. Casciola-Rosen et al have demonstrated that Mi-2 and Jo-1 expression is increased in certain tumours including breast and lung adenocarcinomas (107). Okada et al also demonstrated the presence of autoantibodies to the DNA mismatch repair protein PMS1 (248), a further myositis autoantigen in patients with pancreatic adenocarcinoma, with protein levels increased in the corresponding lesional tissue (249). Furthermore, it has been demonstrated that tumour cells express low levels of specific effector caspases (250, 251), leading to the suggestion that with certain forms of cancer there may be a relative increase in other proteolytic enzymes, including granzyme B. It is therefore plausible that there may be generation of uniquely cleaved autoimmune fragments in carcinoma cells. Suber et al have suggested a hypothesis that in certain circumstances, the autoimmune response in myositis is secondary to a primary anti-cancer response. A further trigger, such as a muscle-trophic virus then leads to enhanced expression of autoantigens promoting activation of autoreactive lymphocytes and tissue damage (138). This theoretical model is supported by Mimuro et al, who demonstrated that the cancer-associated anti-p53 antibody is also observed in PM and DM patients, even when not associated with malignancy (252). The discovery of the p155/140 autoantigen and its clear association with cancer in adult DM is an exciting breakthrough, and may increase our understanding of the relationship between autoimmunity and cancer (143, 157, 158). What is fascinating is the fact that the same protein is an autoimmune target in JDM, which does not appear to be associated with malignancy (see Chapter 6) (161), and this also appears to be the case in younger adults with amyopathic DM (as described in Chapter 4). Perhaps some perturbation of p155/140 in proliferating cells combined with a more efficient anti-cancer response by a younger immune system may be the answer. Interestingly p155 (TIF1-γ) has been shown to inactivate Smad-4, which regulates TGF-β signalling, thus promoting cell growth and differentiation (including malignant tumours) (253). Further studies to investigate p155/140 autoantigen expression and conformation in cancer tissue and compare this to levels in other lesional tissues may be revealing. Whilst the anti-p155/140 autoantibody is the only MSA significantly associated with cancer there is evidence for roles of other autoantigen targets in malignant processes. These include autoantigenic members of the aminoacyl tRNA synthetase family; tyrosyl-, isoleucyl-, phenylalanyl- and glycyl-tRNA synthetase. Of particular interest is the preferential expression of the interstitial lung disease associated autoantigen, phenylalanyl-tRNA synthetase (alpha-subunit) in solid lung tumours and acute myeloid leukaemia (254-260). In addition, MORC3 (NXP-2 / p140) is essential for
regulating the sub-nuclear localisation of p53 (261), and MDA5 activates IkB (IKK) related kinases, known to have a role linking chronic inflammation and cancer (262). The next question of whether MSAs are simply epiphenomena or directly linked to pathogenesis remains uncertain. Two recent publications have highlighted the potential role of anti-Jo-1 autoantibodies in disease activity and pathogenesis. Stone et al investigated the association between anti-Jo-1 levels and myositis disease activity (263). The authors performed a cross-sectional study of 81 anti-Jo-1 positive patients and disease activity was assessed using CK levels and the myositis disease activity assessment tool. Anti-Jo-1 levels were measured using a commercial ELISA and a custom ELISA using recombinant human autoantigen. Anti-Jo-1 levels showed modest correlation with CK, myositis and joint disease activity. Longitudinal data was available on 11 patients that showed serial anti-Jo-1 levels did correlate with CK and disease visual analogue scales. In the second study, Eloranta et al concluded that immune complexes containing either anti-Jo-1 or anti-Ro in the presence of RNA may act as endogenous inducers of type 1 interferon alpha (IFN-α) (80). Together, these findings suggest that anti-Jo-1 autoantibodies may play a role in disease propagation, and it would be of interest to see whether other MSAs correlate with disease activity and are able to induce pro-inflammatory cytokine activity. Therefore in IIM, disease propagation and/or disease flares may be autoantibody driven in an already primed pro-immune environment, which leads to autoantibody mediated autoantigen capture, presentation and opsonisation.

It now appears clear that in IIM specifically targeted autoantigens share unique properties, are preferentially expressed in disease-associated tissues and have a role in disease initiation and propagation. Investigating the structure and function of target molecules, and whether autoantibodies themselves have functional roles appears critical for understanding the pathogenic mechanisms in this complex spectrum of diseases. This in turn, may lead to therapeutic advances including the development of more targeted treatments.
7.2.3 Hypothetical models of autoreactivity and pathogenesis in myositis subsets

Figure 25: Summary of observations: autoantigens as clues in target tissues

- The Mi-2 protein is enriched in DM muscle in comparison to PM muscle and normal muscle (107).
- In mouse models, the Mi-2 protein plays a critical role in the development of the skin basal epidermal layer (134).
- In vitro, UV light upregulates Mi-2 expression in human keratinocyte cell lines (264).
- The Mi-2 protein is enriched in adenocarcinoma tissue (107).
- Expression of the Jo-1 protein is highest in the lung in comparison to other tissues including muscle (108).
- The Jo-1 protein is highly susceptible to granzyme B cleavage in the lung and the immunogenic form of Jo-1 in vivo exists unbound (108).
- Mice immunised with the Jo-1 protein develop the muscle inflammation and lung fibrosis synonymous with the anti-synthetase syndrome (133).
This model is based on the observation and works of others (80, 103, 104, 106, 108, 135, 137, 265-267). An environmental trigger such as a lung trophic virus and/or an environmental toxin (e.g. smoking leading to damage of the lung epithelial barrier) may initiate the autoimmune response in genetically susceptible individuals (e.g. HLA-DR3 haplotype). Virus particles incorporate into the alveolar epithelial cells triggering cytotoxic CD8 T cell mediated release of the serine protease granzyme B. This leads to alveolar cell apoptosis and thus modification of the Jo-1 protein via granzyme B cleavage. Granzyme B generated fragments of Jo-1 become immunogenic. Jo-1 is chemo-attractant via CCR5 receptor binding, thus attracting CD4/8 T cells, monocytes and immature dendritic cells (DCs). The Jo-1 autoantigen may be incorporated into DCs and then presented to CD4 T cells, which in turn initiates T cell autoreactivity and autoantibody production by B cells. This mechanism is enhanced by anti-viral type 1 IFN responses leading to DCs maturation and further antigen presentation. Anti-Jo-1 autoantibody production is also promoted through the interaction of mature DCs with autoreactive B cells via BAFF. This scenario is supported by the finding that bronchial-alveolar fluid from NSIP lung (the most common subtype of interstitial pneumonia in Jo-1 myositis) is rich in CD8 T cells. Therefore, the initiating target tissue in Jo-1 ASS may be the lung with muscle and/or skin targeted at later stages of injury as Jo-1 autoantigen
levels and corresponding autoantibody production increases. It is likely that the other ARS proteins, in particular PL-7 and PL-12 share similar immunogenic properties and expression profiles in the lung, particularly because lung damage is the predominant feature of this subtype of anti-synthetase syndrome.

**Figure 27: Skin model: Initiation of autoimmunity in Mi-2 dermatomyositis syndrome**

In genetically susceptible individuals (e.g. DRB1*07 in Mi-2 syndrome), UV-light triggers cutaneous inflammation. CD8 T cell mediated keratinocyte cell death leads to upregulation of unique immunogenic fragments of the Mi-2 protein. It is possible that the Mi-2 autoantigen also has chemoattractant properties (similar to the Jo-1 autoantigen), thus recruiting T helper cells and DCs. Antigen presentation promotes T cell autoreactivity and anti-Mi-2 autoantibody production by B cells. This process is further enhanced by UV-light induced IFN production leading to DCs maturation and endothelial cell activation. Finally, loss of normal Mi-2 function disrupts basal epidermal cell layer repair mechanisms propagating epidermal damage. Similar to the Jo-1 lung model, attack on muscle may be a secondary downstream effect in
this model. The p155/140 protein or other DM-specific autoantigens such as SAE may be modified by similar mechanisms.

**Cancer model: as proposed by Suber et al (138)**

It is proposed that perhaps in some patients with cancer-associated dermatomyositis the first event in the pathogenic cascade is the initiation of an anti-tumour response. In the early stages of abnormal cell differentiation, the immune system recognises highly expressed tumour antigens (based on current observations these antigens may include Mi-2 and p155/140). This response is either highly effective leading to efficient removal of tumour cells (before any pathological and clinical manifestations) (perhaps this mechanism may explain why p155/140 is a major autoimmune target in JDM) or ineffective (i.e. development of cancer in an ‘older’ less efficient immune system). This powerful anti-tumour response combined with other stressors e.g. toxins or trophic viruses leads to muscle or cutaneous injury. For example, muscle cells may undergo apoptosis, and those antigens initially targeted by the anti-cancer response, become upregulated in areas of repair and regeneration. This in turn leads to antigen presentation, autoreactive T cell propagation and autoantibody production. Inflammatory cells and cytokine production promote further injury and thus repair, which sustains the autoimmune response against cancer, muscle and/or skin cells. With the discovery of the p155/140 autoantigen, this hypothesis can now be interrogated.
7.3 **Future Work**

Future work should focus on increasing our understanding the significance of novel protein targets, from how they are expressed at a cellular level to how they classify patients based upon clinical features.

7.3.1 **Screening larger cohorts of IIM patients to further define clinico-serological subsets**

Further screening is required to establish the prevalence and disease specificity of novel autoantibodies in a European-wide cohort of adult patients with IIM. With the formation of the European Myositis Consortium, cohorts of adult patients with IIM with linked clinical data and serum samples are now available for analysis. This combined with patients recruited to the UK AOMIC Registry and UK JDM cohort study will form the largest collection of adult and juvenile IIM patients (over 1000 patients) with comprehensive clinical data (see chapter 2, appendix, proforma 3 and 4). MSAs are associated with clinical phenotypes and the study of a large cohort of patients will form a major biomarkers resource. Rather than diagnose patients with PM or DM, the work described in this thesis and future studies will lead to defining IIM patients into clinico-serological syndromes, which in the future will aid physicians to predict clinical outcomes and plan specific treatment regimes.

7.3.2 **Investigate the persistence and level of anti-p155/140 in JDM in relation to increasing age and clinical course**

Around 20% of children with JDM are positive for anti-p155/140 autoantibodies. The same autoantibody specificity is found in adult DM associated with malignancy. Therefore, it will be important to investigate how these autoantibodies fluctuate over disease course (disease activity and response to treatment). Serial samples on children recruited to the UK JDM Cohort Study have been stored during disease course annually will be screened for anti-p155/140 using IPP. Autoantibody levels can be quantified using immunoprecipitation blotting. Briefly, immunoprecipitations will be completed using 40μl serum (p155/140 and control serum), 2mg protein A Sepharose and 5 mM bis-(sulphosuccinimidyl)-suberate cross-linker. Beads will be incubated in 2ml unlabelled K562 cell extract for a total of 2 hr at 4°C. Samples will be resuspended in 80μl SDS sample buffer and are heated. Proteins are fractionated by 10% SDS-PAGE and transferred to nitrocellulose by Western blotting. Blots will be probed with anti-p155/140 positive sera and bands will be detected using an alkaline phosphatase conjugated donkey anti-human IgG antibody and the BCIP/NBT liquid substrate solution. The immunoblots will then be
quantified by densitometry. Equal loading will be confirmed by spiking samples with a pre-defined amount of a lower molecular weight protein. Blots will be stripped and re-probed for this protein. Autoantibody data will be linked to prospective clinical data collected every six months and recorded on the JDRF database.

7.3.3 Antigen expression

Lesional tissue

As described in section 7.2.2, based on the paradigm suggested by Casciola-Rosen and Rosen that in IIM lesional tissue is enriched for certain autoantigens that, under certain circumstances, may drive a dysregulated autoimmune response, further work is required to investigate this hypothesis. IIM is an excellent model to explore this concept further. Muscle, lung, skin and cancer biopsy material is readily accessible tissue that can be studied directly. Tissue expression can be investigated by immunohistochemistry - biopsy specimens (stored at -80°C) will be cryostat (6-8 um thick), mounted and dried. Slides will be warmed at room temperature, fixed in ice-cold acetone and air-dried. Sections will be washed in PBS and rinsed in TBS-tween. Sections will be blocked and incubated overnight at 4°C in a humidified atmosphere with commercial monospecific primary antibodies to myositis specific antigens (SRP54 chicken polyclonal ab14072, Mi-2 rabbit polyclonal 06-878, TIF1-γ mouse monoclonal ab33475, SAE1 sheep polyclonal alx210328, SAE2 rabbit polyclonal ap10659, HARS mouse monoclonal ab50835, TARS mouse monoclonal ab50147, ALARS rabbit polyclonal ab50147, NARS rabbit polyclonal ab50144, GARS rabbit polyclonal ab42905, Isoleucyl tRNA-synthetase rabbit polyclonal ab31533, Phenylalanyl tRNA-synthetase rabbit polyclonal ab42905, Phenylalanyl tRNA-synthetase beta H00010056-A01 mouse polyclonal, Phenylalanyl tRNA-synthetase alpha H00002193-A01 mouse polyclonal. Sections will be washed and incubated with the appropriate secondary antibodies (anti-mouse/rabbit/sheep/chicken IgG) conjugated with horseradish peroxidase, stained with 1-Step 4-CN (Perbio) and visualised. Negative controls will be performed by omitting or isotype-matching with the primary antibody. Sections will also be probed with FITC and TRITC conjugates as secondary antibodies in order to co-localise autoantigens by double immunofluorescence with appropriate cell types present in lesional tissue. IPP and immunoblotting on cell extracts will be performed to ascertain the specificity of commercially prepared antibodies prior to immunohistochemistry (as already done with commercial anti-SAE1/2 and anti-NXP-2 demonstrating that these antibodies do recognise corresponding antigen targets derived from human cell lines.
Different cell lines

Although the autoantigen targets can be visualised using \[^{35}S\], for characterisation with mass spectrometry it may be necessary to scale up the quantity of polypeptide immunoprecipitated (e.g. if it is constitutively expressed in low copy number). The initial approach will be to increase the quantity of protein-A-Sepharose beads and source of antigen. However, subsequently, a combination of sub-cellular fractionation and immunoaffinity chromatography may be used to optimise antigen recovery. In addition, other human cell lines may contain different levels of expressed known and novel myositis autoantigens. Differences of expression in these seemingly ubiquitously expressed proteins will provide additional insight into pathogenic mechanisms.

7.3.4 Development of quantitative solid-phase assays

The future development of commercial assays that test for MSAs in routine clinical practice is important. To date, certain myositis autoantigens (Jo-1, PL-7, PL-12, Mi-2 and SRP) have been expressed as recombinant proteins for use in commercial ELISAs and line-blots. An important aim is to develop further assays for the novel MSAs including the detection of anti-p155/140 (p155 autoantigen - TIF1-\(\gamma\) (159)), given the potential importance of this autoantibody in the diagnosis of JDM and its association with malignancy in adult-onset DM. Furthermore, the development of custom-based ELISAs will enable the quantitative analysis of MSA levels in serum samples that can be correlated with measures of disease activity, as previously demonstrated with anti-Jo-1 autoantibodies (263). Other autoantigen systems that may also become strategically relevant include anti-SAE, anti-p140 and anti-CADM-140. To develop sensitive immunoblotting techniques and custom based ELISAs preparations will be enriched with the appropriate autoantigens by a combination of cell fractionation, HPLC and affinity purification using monoclonal antibodies. An alternative approach is to extract mRNA from cell lines e.g. K562, A549 cells to develop a cDNA library. This will be screened by either PCR or using a SEREX. Positive sequences will be expressed in a suitable system for the large-scale production of the recombinant peptide. In addition, commercial supplies of the relevant autoantigen can be sourced e.g. TIF1-\(\gamma\) peptide (AbCam 47108) that will be tested for autoantibody recognition by immunoblotting using a panel of appropriate positive and negative controls. A further approach will be the generation of 20-mer overlapping synthetic peptides spanning the known sequence of autoantigens.
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Invited Contributions and Selected Case Reports


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